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THE UNIVERSITY OF ALBERTA

Studies on the Biosynthesis and Mechanism of Action of Polyene Antibiotics

by

© Paul Hedley Morrell Harrison

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Department of Chemistry

EDMONTON, ALBERTA

Fall 1987

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Supervisor

External Examiner

Date May 28, 1987.

To my wife, Colleen

Abstract

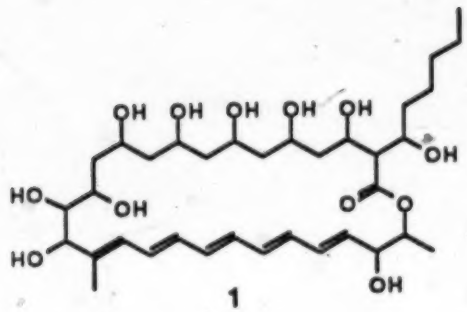
The biosynthesis and sterol binding properties of fungichromin (1), a typical member of the polyene group of antibiotics, were investigated. Polyene antibiotics are used in the treatment of human fungal diseases; current theory holds that they act by binding of sterols in membranes thereby releasing cell components and causing cell death. Preferential binding to ergosterol over cholesterol has been proposed to account for the selectivity of polyene toxicity to fungal cells, but actual structures of the complexes are unknown.

Incorporation of sodium [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-acetates, [1-^{13}C]propionate, and [1-^{13}C]- and [3-^{13}C]-octanoate showed that **1**, a metabolite of *Streptomyces cellulosae*, derives from 1 propionate, 12 acetate, and one intact octanoate units, condensed in the head-to-tail fashion typical of polyketide biogenesis (in collaboration with Dr. H. Noguchi). The results with octanoate are the first case of incorporation of a fatty acid with more than 4 carbon atoms as a unit into a polyketide without detectable degradation. Incorporation of ethyl [CD_3]oleate gave specifically labelled **1** (in collaboration with Dr. K. Arai): octanoate thus derives biosynthetically from oleate. Incorporation of sodium [1-^{13}C, $^{18}O_2$]-labelled acetate, propionate and octanoate, and $^{18}O_2$, gave **1** in which the labelling pattern was consistent with polyketide biogenesis. Diethyl [2-^{13}C]malonate was incorporated into **1** as acetate. Efficient incorporation of a mixture of diethyl [2-^{13}C]- and [1,3-$^{13}C_2$]-malonates gave **1**, the 2D-INADEQUATE spectrum of which gave carbon-carbon connectivities complementary to those obtained from incorporation of sodium [1,2-$^{13}C_2$]acetate. This novel technique gave full assignments of the ^{13}C NMR spectrum of **1** for the positions derived from acetate.

To determine sterol binding structure-activity relationships, cholesta-5,7-dien-3β-ol, (22*E*)-cholesta-5,22-dien-3β-ol, (22*E*)-cholesta-5,7,22-trien-3β-ol, (22*E*)-ergosta-5,22-dien-3β-ol, ergosta-5,7-dien-3β-ol, and ergost-5-en-3β-ol were synthesised, but a UV assay showed little difference in binding to **1** between these sterols.

Photoaffinity labelled sterols, 3β-(2'-diazo-3',3',3'-trifluoropropionyloxy)-cholest-

5-ene (2) and (20*S*)-20-(2'-diazo-3',3',3'-trifluoropropionyloxymethyl)-pregn-5-en-3β-ol (3) were synthesised: 3 binds to 1 in the UV assay, but 2 does not. However, photolysis of 3 in the presence of 1 failed to give labelled products. Both 7α- (4) and 7β-acetoxycholesterol (5) were synthesised: 4 binds to 1 but 5 does not, suggesting that a flat sterol B-ring is not the only requirement for efficient binding to polyenes. Synthetic progress towards sterols with a photoaffinity label at C-7 is described.



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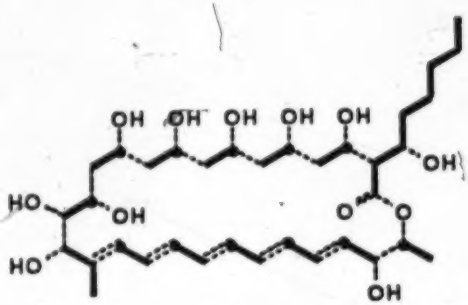

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List of Abbreviations

Ac	CH_3CO
APT	attached proton test
Bu	butyl
CI	chemical ionization
CoA	coenzyme A
COSY	correlated spectroscopy
DCC	1,3-dicyclohexylcarbodiimide
DMAP	dimethylaminopyridine
DMPC	dimyristoylphosphatidylcholine
DMSO	dimethylsulfoxide
egg-PC	egg yolk phosphatidylcholine
EI	electron impact ionization
Enz	enzyme
Et	ethyl
FAB	fast atom bombardment
FID	free induction decay
GC	gas chromatography
HPLC	high performance liquid chromatography
INEPT	insensitive nucleus enhancement by polarisation transfer
IR	infrared spectroscopy
K_{app}	apparent equilibrium constant of binding
LDA	lithium diisopropylamide
Me	methyl
MPLC	medium pressure liquid chromatography
MS	mass spectrometry

NMR	nuclear magnetic resonance
n.O.e.	nuclear Overhauser enhancement
pent	pentuplet
Ph	phenyl
ppb	parts per billion
Pr	propyl
Py	pyridine
SEFT	spin echo Fourier transform
TBDMS	*tert*-butyldimethylsilyl
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
Ts	*p*-toluenesulfonyl
χ	denotes a single, but unknown stereochemistry
$\hat{\chi}$	stereochemistry opposite to χ

I. Introduction

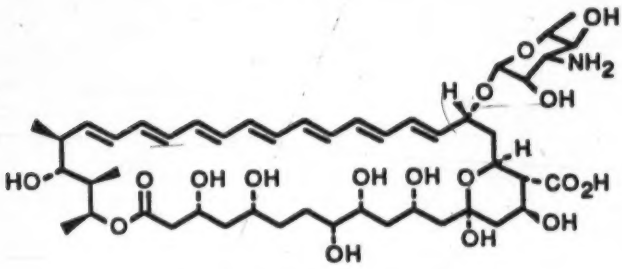
General Introduction

The polyene antibiotics[1, 2] are a group of over 200 compounds, produced by *Streptomyces* species, that possess antifungal and antiprotozoal activity.[3] Despite their acute toxicity,[3-6] and the development of other classes of antifungal antibiotics,[7, 8] the polyenes remain the treatment of choice for many fungal infections.[3, 7] Recently, the possible antihypercholesterolemic effects of polyenes[4, 9, 10] have attracted considerable interest, as has their potentiation of the anti-tumor properties of other drugs.[11-14] Polyenes may also have some antiviral activity, either alone[15] or in combination with other drugs.[16] Some polyenes are used as food preservatives; for example, pimaricin protects blue cheese from fungal growth.[17]

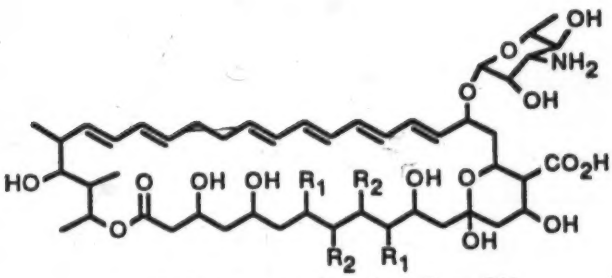
Chemically, these steroid-binding[18] compounds, for example amphotericins A and B, nystatin A_1, partricins A and B, pimaricin, fungichromin (1) and filipin III (2) (Figure 1), possess the common feature of an extended conjugated polyene chain of 3 to 8 double bonds within a macrocyclic lactone ring.[1] Complementary to the polyene chain, along the other side of the lactone ring, is a saturated carbon chain adorned with hydroxyl groups. In some polyenes, the sugars mycosamine or perosamine (Figure 1), or an aromatic moiety, *p* -aminoacetophenone (e. g. partricin B) or N-methyl-*p* -aminoacetophenone (e. g. partricin A), may be present as pendants on the macrocyclic ring.[19]

The polyenes absorb very strongly in the ultraviolet. The absorption maxima depend on the length of the polyene chain, thereby allowing easy partial characterisation.[19] They are therefore normally categorised by number of conjugated double bonds present. Thus amphotericin B is a heptaene, and fungichromin (1) is a pentaene.

Figure 1A. Structures of some common polyene antibiotics.

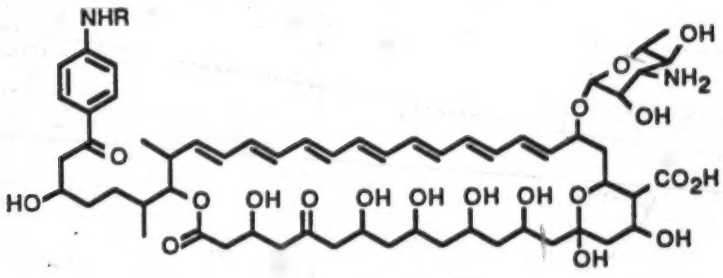


Amphotericin B



Amphotericin A , R_1 = H, R_2 = OH

Nystatin A_1 , R_1 = OH, R_2 = H

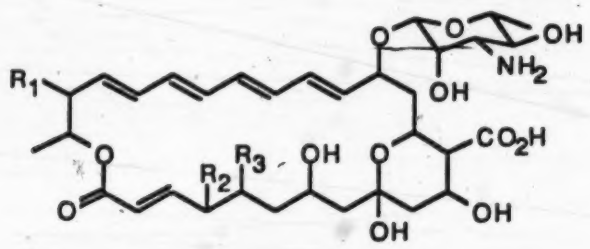


Partricin A, R= Me

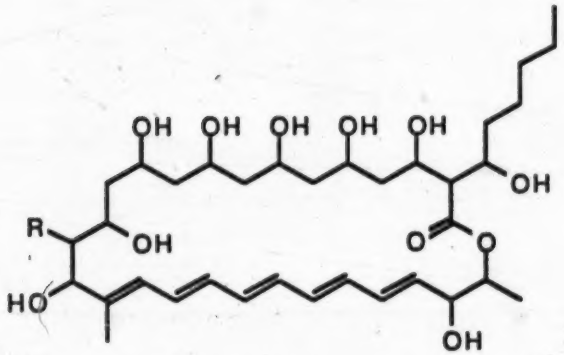
Partricin B, R= H

Nystatin was the first polyene to be isolated,[20] and it, along with partricin methyl ester (tricandil^{TM}),[21] is used for topical creams for surface or vaginal fungal infections.[3] Amphotericin B[22, 23] is the drug of choice for deep-seated internal mycotic infections.[3, 7] First isolated in 1955,[24] the compound was shown to have strong antifungal[25-27] but no antibacterial[27] activity.

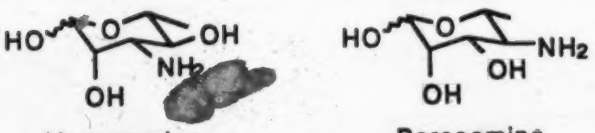
Figure 1B. Chemical structures of some common polyene antibiotics.



Pimaricin, R_1 = H, R_2, R_3 = epoxide
Tetramycin A, R_1 = Et, R_2 = H, R_3 = OH
Tetramycin B, R_1 = Et, R_2 = OH, R_3 = H



1, fungichromin, R= OH
2, filipin III, R= H



Mycosamine Perosamine

Early chemical studies by Dutcher *et al*[28] showed that amphotericin B contained the amino-sugar mycosamine; titration and analysis gave an approximate molecular weight, and a tentative formula was proposed. Most significantly, catalytic hydrogenation led to uptake of 7 moles of hydrogen, to give a biologically inactive product. This result showed that the polyene chromophore is important for activity, and thus opened up the search for novel polyenes. Filipin,[29] fungichromin,[30] and a host of other polyene antibiotics were

rapidly discovered.

Further early work on the chemical degradation of amphotericin B by Mechlinski and coworkers[31-34] and Cope *et al*[35] established the partial structure of the antibiotic, and culminated in the elucidation of the full atomic connectivity in 1970.[36] At the same time, Mechlinski and coworkers[37, 38] also published the X-ray crystal structure of N-iodoacetylamphotericin B. The compound exists as a hemiketal; this result has been confirmed in solution by ^{13}C NMR,[39] optical rotatory dispersion,[40] and IR spectroscopy.[41] However, IR spectra of amphotericin B show ambiguities that depend on the method of sample preparation.[42] Conformational calculations show that the molecule is rigid.[43]

Despite extensive studies, amphotericin B remains the only polyene for which the absolute configuration at all the chiral centres is known. Some other polyenes have not yet been assigned full structures. Amphotericin A,[44, 45] nystatin,[46, 47] partricins A and B,[48] filipin and fungichromin,[49] and pimaricin,[50] have well defined structures. The difficulties associated with purification of the highly insoluble compounds caused early ambiguities; materials that appeared to be distinct were often shown to be identical upon further purification. For example, fungichromin is identical to lagosin and cogomycin;[51] nystatin, thought for some time to be identical to amphotericin A,[47] was shown to be distinct;[45] while hexaenes endomycin and hexafungin each contain 2 identical major polyenes.[52] These comparisons have been facilitated by the gradual development of TLC[21, 22, 39, 51-53] and HPLC[22, 45, 51-54] systems for polyenes. HPLC has also proved useful for analysis of antibiotic levels in man during treatment.[4, 5] Countercurrent distribution has proved the preferred method for purification to a high level.[21, 51, 55]

Structural elucidation has been aided by the advent of modern techniques. Early studies of the electron impact mass spectra of polyenes relied upon per-trimethylsilylation: although molecular ions were not observed, comparison of fragmentation patterns aided in structure elucidation and comparison of identities.[22, 45, 56] More recently, use of field-

desorption,[55, 57-59] plasma desorption,[45] and fast-atom bombardment[21] mass spectra, has allowed direct determination of the molecular weights of polyenes.

The use of high-field-, and recently multidimensional, NMR has also greatly aided analysis and structure elucidation of polyenes. Carbon-13 NMR is particularly powerful, as it is sensitive to differences in the unknown stereochemistries of polyenes,[45, 51] and permits determination of such features as ketalisation in solution.[21, 39] Two-dimensional NMR has allowed full proton assignment of nystatin,[60] confirmed the structure of tetramycin A,[59] and determined the structures for tetramycin B[59] and amphotericin A.[45] Development of NMR and mass spectrometric methods for structural elucidation, and chromatographic methods for purification, is thus an important area in polyene research.

Despite the high potency and therapeutic index of amphotericin B,[26] many difficulties are encountered in treatment with the drug.[3] The compound is highly toxic, patients require hospitalization for treatment, and it may cause permanent kidney damage. A further problem is the very low solubility. Therefore, treatment may be effected by slow intravenous infusion of amphotericin B dissolved in DMSO, and then made into a suspension in water. While use of solubilising agents such as sodium desoxycholate[61] (fungizone™ from Squibb is the combined amphotericin B-desoxycholate mixture) and other cholate analogues[62] is now the method of choice for administration, the search for more water-soluble chemical derivatives continues. The free carboxyl group of amphotericin B has been esterified,[63] usually to the methyl ester using diazomethane.[64-66] The derivative has full biological activity,[66, 67] and gives a very water-soluble hydrochloride.[65] However, more recent work suggests that the stability of the methyl ester is lower than that of the parent antibiotic,[68] and describes a lower biological activity, at least against *N. fowleri*.[69] In some cases the product has been shown to be a mixture containing amphotericin B methyl ester and N- and O- mono- and per-methylated analogues.[66]

In contrast, the N-acyl derivatives[64, 70] have distinctly lower biological activity:

N-iodoacetylamphotericin B,[64] used for the X-ray crystal structure,[37, 38] has a minimum inhibitory concentration between 1 and 20 times that of the parent compound against *Saccharomyces cerevisiae* , *Aspergillus niger* and *Candida albicans* .[64] Other derivatives prepared include N,N-dialkyl derivatives,[72] amides attached at the carbonyl group,[57] guanidine-type derivatives attached at the amino group,[6] derivatives formed by attachment of extra sugar moieties,[73-75] and trimethylammonium salts.[55, 71] The latter are water-soluble, are as active as the parent polyene, and show reduced haemolysis of erythrocytes, which is indicative of reduced toxicity. None of these derivatives appear to have yet been used parenterally in general clinical practice, however, despite extensive patent applications.

Biosynthetically, the macrocyclic ring of polyenes derives from acetate and propionate.[19, 76] Birch *et al*[77] first found good incorporations of [^{14}C]-labelled acetate and propionate into nystatin aglycone. Perlman and Semar[78] used incorporation of [2-^{14}C]acetate into amphotericin B to prepare the radiolabelled drug for clinical studies. Many other similar results have been reported[19] for amphotericin B,[79] lucensomycin,[80] candicidin,[81, 82] fungimycin[83] and levorin.[84] The terpenoid precursor, [2-^{14}C]mevalonic acid,[77] and [*methyl*-^{14}C]methionine,[77, 78, 83] do not label polyenes. Tritiated acetate ($CTH_2CO_2^-$) has been used to prepare tritiated amphotericin B.[66, 85] Inhibition of polyene production by cerulenin, a known inhibitor of fatty acid synthase, has been interpreted as evidence for a polyketide biogenesis.[19] Due to difficulties associated with using antibiotic degradation to determine the sites of labelling,[77] the sum evidence for polyketide biosynthesis, though strong, is somewhat indirect, and little work has been done in this area in the last 15 years. No NMR studies of polyene biosynthesis using stable isotope labelling techniques have been reported, with the exception of that described in this thesis.[86]

Co-production of polyenes by one organism may indicate a similar biosynthetic path. Conversion of amphotericin B to amphotericin A via a cyanide-inhibited reductase has

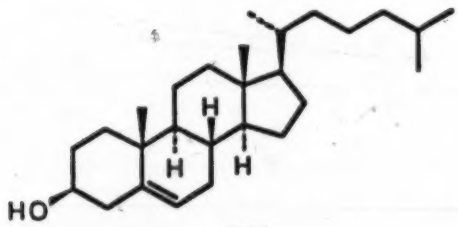
been demonstrated by Liu.[66]

The biosynthetic origin of the aromatic and sugar moieties in polyenes has been much more extensively investigated, and is the subject of several reviews.[19, 87]

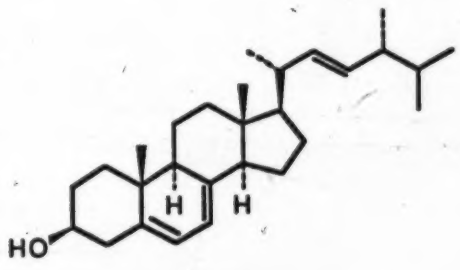
Mechanism of Action

Extensive evidence indicates that steroids play a major role in the action of the polyene antibiotics.[18, 23, 88] This section presents a summary of this evidence.

Erythrocytes (red blood cells),[89-95] which contain large amounts of cholesterol (3), and fungi,[90, 92-96] which contain other sterols, often ergosterol (4), are sensitive to polyenes. However, bacteria, which contain little or no sterol, are insensitive.[27] Addition of exogenous sterol to the medium protects sensitive cells.[90, 95, 97-99] In contrast, cells grown in the presence of sterol, which thus have higher sterol levels, are more sensitive.[89, 96, 100, 101] Resistant mutants and varieties of a species that is normally sensitive to polyenes have lower sterol levels[102, 103] or different sterols.[104, 105] Compactin, an inhibitor of cholesterol biosynthesis,[106, 107] protects chinese hamster cells against damage by polyenes.[108] Amphotericin B inhibits esterification of cholesterol in plasma.[109]



3
Cholesterol



4
Ergosterol

These biological observations suggest that a complex may form between sterol and

polyene. The most direct evidence for this binding comes from spectral changes in the polyene upon addition to sterol suspensions in water.[110] Such changes have been observed in the ultraviolet (UV),[89, 90, 99, 111- 116] fluorescence,[117, 118] and circular dichroism (CD)[112, 115] spectra of polyenes. Organic solvents destroy the complex.[111] The situation is complicated by extensive aggregation of polyenes in the aqueous phase, as shown by UV[119-123] and CD[119-121, 123-125] spectra. The spectra show strong coupling of the chromophores between molecules, indicative of micelle formation with the hydrophobic polyene chains close together in the interior. In contrast, in anhydrous organic solvents, 'inverted' micelles are formed.[116] The UV[115, 116, 122] and CD[115, 116] spectra of polyenes bound to sterols in aqueous suspensions are more similar to the spectra in organic solvents. This has been interpreted as indicative of a hydrophobic interaction between polyene and sterol.

That such observations are of relevance to the biological situation has been shown. *Acholeplasma laidlawii* (*Mycoplasma laidlawii*) is an organism that grows without de novo synthesis of sterols. However, addition of sterol to the growth medium results in its incorporation into the organism. UV spectra of polyenes added to this species grown in the presence of sterol show changes very similar to those in the *in vitro* experiments.[89, 126] Erythrocyte ghost cells (the membranes of erythrocytes) show a similar effect on the UV spectrum.[89] Liposomes from *Candida albicans* affect the CD spectrum in the same way as sterol suspensions, but only for sensitive strains.[105]

One important role of sterol in cells is the effect on the cell membrane. Freeze-fracture and freeze-etch electron microscopy show that polyenes influence the membrane structure of cells, forming 'pits' and 'ridges' on the formerly relatively smooth cell membrane.[126, 127]

The most extensive spectral studies have been performed on liposomes or vesicles, which provide simplified models for the complex sequences of events occurring during polyene action. Equilibrium binding of polyene to sterol has been examined by UV,[89,

114, 118, 127-133 CD[127, 134-136] and fluorescence.[117, 121, 127] In the case of fluorescence, amphotericin B is reported to give negligible changes upon binding sterol in one case.[137] UV has been used to investigate the kinetics of binding.[118, 128, 129, 132] Ockman used polarised absorption[138] and reflection[139] spectroscopy of egg lecithin-cholesterol monolayers on water to demonstrate interaction of the sterol with amphotericin B. Gel permeation chromatography has been used to show binding of polyenes to cholesterol-containing vesicles.[140]

However, the investigation of the interaction in such models of membranes is complicated by several factors, such as the aggregation state of the polyene described above. Furthermore, UV studies on addition of polyenes to fatty acids in water show complexing effects similar to, though weaker than, those observed with sterols.[141] Analogous studies with sterol-free vesicles,[121] as well as CD spectra,[125] show that interaction occurs: the extent of interaction with fatty acids depends on vesicle size and fatty acid structure.[133] Fatty acids also protect *Saccharomyces cerevisiae* against polyenes.[141] Raman spectroscopy provides further evidence for complexation.[142]

In another case, transfer of amphotericin B from egg-phosphatidyl choline (egg-PC) vesicles to dimyristoylphosphatidyl choline (DMPC) vesicles was demonstrated.[134] The reverse process did not occur. The results were interpreted in terms of preferential binding to the DMPC system, which is in the gel state, over the egg-PC system, which is in the liquid-crystalline state.

Other evidence suggests that the polyenes alter the ordering of the lipid bilayer. The liquid-crystalline to gel phase transition temperature of lipids is altered by sterols, which confer rigidity upon the membrane. This effect of sterols is reversed upon addition of polyene, as has been shown by differential scanning calorimetry.[89, 114] Further evidence for disordering of the lipid bilayer by antibiotic comes from the loss in sharpness of the choline methyl resonances in the proton NMR spectrum of egg-PC vesicles,[140] the work of Smith and co-workers[143] on deuterium NMR of vesicles containing deuterated lipid,

and the use of electron spin resonance (esr) in conjunction with lipids or sterols chemically modified with a substituent which gives a stable free radical.[144, 145]

Although polyenes exert these effects in sterol-free systems, there is a more dramatic effect when sterol is present, both in the deuterium NMR case[146] and in the esr case.[130, 145, 147-149]

Extensive evidence indicates that once bound to the lipid bilayer membrane, polyenes cause leakage of cellular components out of the cell, or materials in the medium into the cell. At high polyene concentrations, cell death occurs, and the cell wall integrity is apparently totally destroyed to give complete release of all components. However, selective release of cellular components occurs at lower antibiotic levels. Potassium ion release[150] has been observed from *Acholeplasma laidlawii* when grown in the presence of sterol,[114, 126, 151] erythrocytes and yeast,[92-94] *Chlorella vulgaris*,[152] and *Candida albicans*.[95] Similar effects were observed in liposomes,[153] as was release of $^{22}Na^{+}$ ions and M^{2+} (M = Cu, Zn, Co, Cd, Ni, Mn, Fe and Pb).[154]

In vesicles, effects ranging from release of H^{+} [134, 155] to influx of ascorbate[156] have been reported. In the latter case, tempo-choline, a spin-labelled molecule, was trapped inside the vesicles during preparation. Monitoring the esr spectrum shows that exogenous ascorbate has no effect until amphotericin B is added. Then, as ascorbate is transported across the membrane, it reacts with the spin probe to destroy the free radical. In another interesting experiment, vesicles containing dicetyl phosphate were placed in a solution of Pr^{3+}. The lanthanide causes a paramagnetic shift in the ^{31}P NMR signals only for the phosphate groups on the outside of the membrane. Upon addition of polyene, Pr^{3+} leaks into the cell, causing a shift in the phosphate groups on the vesicle inside layer as well.[157]

Neutral molecules as well as ions may leak from the cells. Small sugars leak rapidly, but glucose only slowly from *A. laidlawii*.[126] Glucose is also released from model membranes.[130, 158] In the *A. laidlawii* experiment, glucose-6-phosphate dehydrogenase was not released by heptaenes, but was released by filipin (2).[126] Release

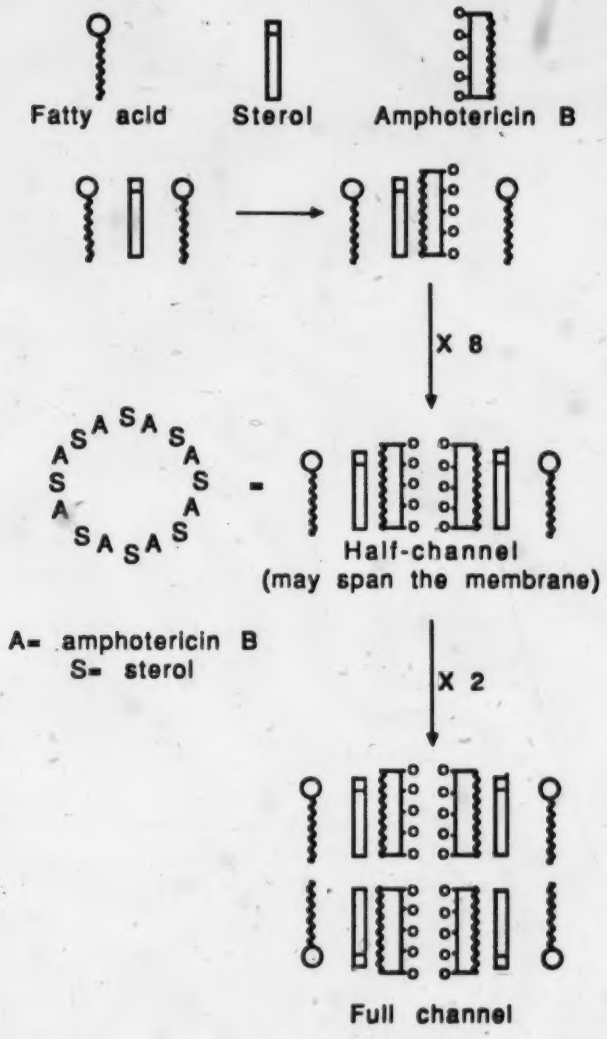
of this large molecule is interpreted as being due to cell death.

In parallel with the transport studies, extensive investigations, largely by Russian groups, have been reported on the conductivity of artificial membranes.[159-162] Addition of amphotericin B to one side of an artificial bilayer containing cholesterol leads to increased conductance, but the effect is much more dramatic when polyene is added to both sides.[163] In the latter case, conducting 'channels' form which exist for finite times in 'open' and 'closed' states.[164, 165] The state of the channel depends on the surface charge on the bilayer,[161] and hence on the ionization state of the carboxyl and amino groups in amphotericin B.[164] More than one type of channel exists.[166] Through study of the time dependence of the conductivity change when amphotericin B was added to one side only of the bilayer, formation of half-channels, that are slowly converted to full channels was shown.[167]

Interestingly, the channels or half-channels may be 'blocked' by addition of tetra-alkylammonium salts such as tetraethylammonium.[168, 169] By varying the alkyl substituents, and measuring their effectiveness as channel blocking agents, it was possible to assess the size of the channel as approximately 7 Å.[170, 171] This result is supported by the blocking effect of various sugars.[171] A theoretical model of the channel blocking process has been reported.[172]

The combined results of these experiments that were then available led De Kruijff and Demel[88] to propose a model for the mechanism of action of amphotericin B in 1974. Polyene-sterol complexes form initially in the membrane. This brings the hydrophilic half of the polyene molecule into the hydrophobic membrane core. To avoid this unfavorable interaction, the polyene-sterol complex oligomerises, allowing favorable interactions between the polyene chain and sterol to occur on the outside. Furthermore, a hydrophilic central region containing exposed hydroxyl groups forms on the inside (Figure 2). This constitutes a half-channel which in some cases may span the entire membrane; in others, two such half channels on opposite sides of the bilayer membrane may coalesce to form a

Figure 2. Formation of pores in the cell membrane by amphotericin B.[88]



full channel. This theory is consistent with the formation of 'mixed channels' having different antibiotics (e. g. nystatin and amphotericin B) on each side of the membrane,[173] as well as with the conductivity results.[163] Ions may then be transported through the channel or membrane-spanning half-channel. Assuming that the size of the channel is approximately that of the glucose molecule, since glucose is about the largest molecule to leak out of cells, the Dutch workers proposed an octameric cyclic half-channel formed from

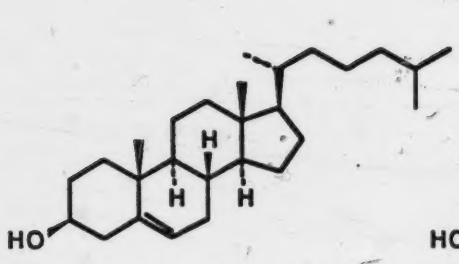
eight polyene and eight sterol molecules (Figure 2). This gives a channel size of 5 Å, in good agreement with the Russian results.[171]

The major differences in nature of binding for filipin and amphotericin B have been interpreted in terms of different channel structures for the two antibiotics in the membrane.[148, 174]

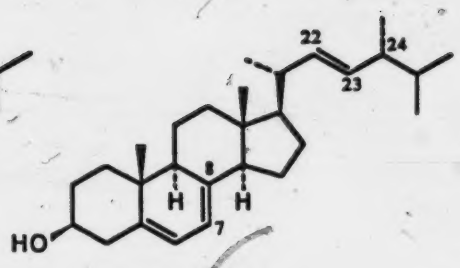
Such a model requires that binding of metal ions by polyenes occur, at least in the complex channel. Direct evidence for polyene-metal interaction has been observed for nystatin by Brown *et al*.[60] Addition of sodium ions to a methanolic solution of nystatin caused changes in the chemical shift of certain resonances in the proton NMR. A change in the ^{23}Na NMR spectrum was also observed upon binding. Considerable indirect evidence for binding of magnesium by polyenes also exists.[175]

Considerable data supports the hypothesis that selective toxicity of polyenes towards fungal over mammalian species is due to preferential binding of the polyene to ergosterol (4) rather than cholesterol (3). For the more toxic polyenes, binding of 3 may be preferred. Many of the studies reported have been discussed above in relation to evidence for the sterol binding hypothesis.[15, 90, 93, 94, 96, 111, 112, 114, 117, 118, 128, 131, 136, 151, 153, 155, 176] The extent of selectivity depends on the polyene and the nature of the system used. Absence of selectivity has been reported in two cases,[130, 177] probably due to the system studied.

Cholesterol (3) and ergosterol (4) differ in three structural features, specifically the presence in ergosterol of double bonds between C-7 and C-8 and between C-22 and C-23, and a methyl group at C-24. In order to determine which structural features in the sterol are responsible for enhanced binding, investigations have been extended to include other sterols.

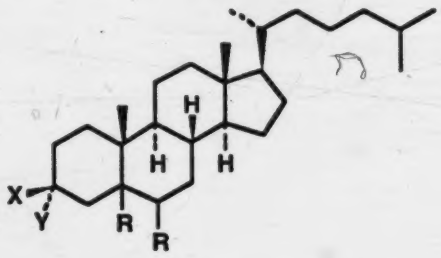


3



4

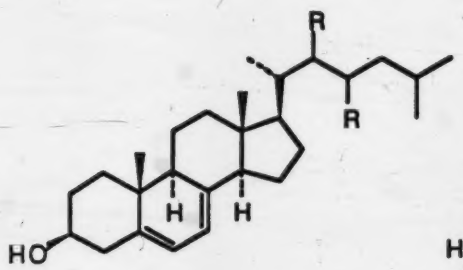
A 3β-hydroxy group is necessary. Thus, 3α-hydroxycholest-5-ene binds much more weakly to filipin (2) than cholesterol (3),[89, 118, 126, 127] while 3β-thiocholesterol[127] and cholestane[95, 104] do not bind. The hydroxy group must be free: cholesterol acetate (5) binds more weakly than 3 both to 2[89] and to amphotericin B,[104] while cortisone acetate also binds less effectively than cortisone.[113] In this cortisone study, which measured interaction by UV with four different polyenes including 2, 3β-hydroxysterols bound more strongly than the corresponding 3-oxo compounds. In contrast, another UV study on 2 reports the strongest binding for cholest-5-en-3-one.[89]



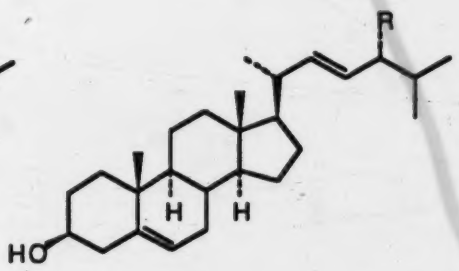
	X= H,	Y= OH,	R,R= bond,	3α-hydroxycholest-5-ene
	X= SH,	Y= H,	R,R= bond,	3β-thiocholesterol
	X=	Y=	R= H,	cholestane
5,	X= OAc,	Y= H,	R,R= bond	
	X,	Y= =O,	R,R= bond,	cholest-5-en-3-one

While these results are in general agreement, studies on selectivity effects upon variation of the sterol double bonds show considerably more disparity. Investigations of cholestanol show that it binds more weakly,[127, 178, 179] approximately equally,[89, 99, 129] or more strongly,[126] than 3 to filipin (2). Introduction of a double bond between C-7

and C-8 to give cholesta-5,7-dien-3β-ol (**6**) results in much stronger binding to amphotericin B than for **3**:[129, 178] it increases the time that channels in an artificial membrane spend in both open and closed states, but is still much less effective in this test than ergosterol.[180] In contrast, with **2**, under conditions where cholesterol binds more strongly than ergosterol, **6** binds more strongly than both.[89] In studies of artificial bilayer conductivity, (22*E*)-cholesta-5,7,22-trien-3β-ol (**7**) was as effective as ergosterol (**4**), and both were more effective than cholesterol, which in turn was approximately as efficient at binding as (22*E*)-ergosta-5,22-dien-3β-ol (**8**).[179] The results indicate that the side-chain plays little role in governing the strength of interaction. This result is supported by the observation that (22*E*)-cholesta-5,22-dien-3β-ol (**9**) and the Z-isomer bind only as well as cholesterol (**3**).[129] However, these results are not necessarily consistent with observations on (22*E*)-stigmasta-5,22-dien-3β-ol: weaker,[89, 99, 113, 126] equal,[95] or stronger,[96, 180] binding compared to cholesterol, has been observed in this case.



6, R,R= H
7, R,R= bond



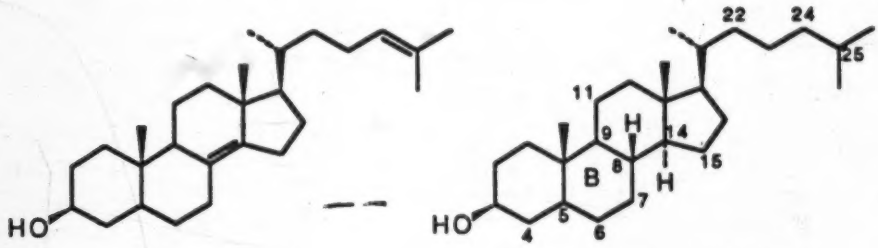
8, R= Me
9, R= H

(22E)-stigmasta-5,22-dien-3β-ol, R= Et

Zymosterol (cholesta-8(14),24-dien-3β-ol) also gives ambiguous results. For protection of *C. albicans* by addition of exogenous sterol, the order of efficacy is zymosterol > ergosterol > cholesterol.[95] However, in experiments studying leakage of Pr^{3+} into vesicles, the order of effectiveness was ergosterol > cholesterol ≈ zymosterol.[157]

Sterols without a side-chain do not bind effectively.[89, 113, 127] Optimal binding kinetics for filipin[129] or amphotericin B[174] are observed for 26-carbon sterols; longer[129] or shorter[129, 174] side-chains, or a 25-hydroxy group,[129] reduce the rate. However, the amphotericin B study found **2** to be unselective in terms of side-chain length.[174]

These results, and others described in the references cited, though complex, do generally illustrate that introduction of more double bonds into the cholestanol skeleton causes increased interaction. However, the location of the double bonds is important: conductivity experiments revealed the sequence -5,7-diene >> -7-ene > -5-ene = -5,8-diene = -7,14-diene = -7,9(11)-diene > -8,14-diene = -ane > -4,6-diene, for the 3β-hydroxycholest- series.[178] General conclusions have been that a flat 'B'-ring is the principal determinant for efficient binding.



Zymosterol — Cholestanol skeleton

The limited availability of sterols possessing varying numbers of double bonds in the 5, the 7 and the (*E*)-22 positions, and with or without the methyl group at C-24, seriously hampers investigation of the role of these functionalities in polyene-sterol binding. Since different techniques or antibiotics give apparently different results, the comparison between these different sterols must obviously be performed under identical conditions, requiring the availability of these compounds simultaneously in one laboratory. Clearly, such an experiment is much needed.

Other studies have investigated structure-activity correlations for the antibiotics. Conductance studies show that nystatin, with a break in the polyene chain, forms channels that are more often 'closed' than those of amphotericin B.[164] In a series of derivatives of

amphotericin B and other heptaenes, permeability and cationic selectivity,[91] potassium release,[93] effect on *S. cerevisiae*,[92] and haemolysis of erythrocytes,[91, 92, 181] have been studied as a function of the lipophilicity of the substituent. The haemolysis and *S. cerevisiae* study shows that the influence of the substituent is not the same in both assays, which may allow rational design of substituents to maximise the therapeutic index.[92]

Despite the large number of results in this area since its publication in 1974, the polyene-sterol binding and channel model of De Kruijff and Demel has not been significantly modified. Although all the techniques described give evidence for a general binding phenomenon between sterol and polyene, no information has revealed the detailed molecular structure of the complex. Examination of molecular models suggests that sterol and polyene align with their long axes parallel. Further, sterol hydroxyl groups in the membrane are known to be situated at the water interface; the sugar-containing polyenes are assumed to insert into the membrane so that the sugar is at or above the membrane surface, close to or in the aqueous phase. However, these restraints still allow a large number of possible sterol-polyene geometries within the complex.

Recently, Professor S. Fraga and Drs. Z. Barandiran, M. Klobukowski and L. Seijo (Department of Chemistry), with computer graphics provided by Professor W. Anderson and Mr. D. Bacon (Department of Biochemistry), (University of Alberta) have undertaken theoretical investigations of the polyene-sterol interaction. Initially, the preferred geometry for one molecule of cholesterol binding to one molecule of amphotericin B was determined. Then, by allowing two such units to interact, a preferred geometry for the dimeric $(\text{amphotericin B})_2$ - $(\text{cholesterol})_2$ species was found. Proceeding in this manner, the investigators were able to show that the preferred oligomer used 8 molecules of amphotericin B and eight of cholesterol to form a cyclic species. This calculation is thus in excellent agreement with the empirical model of De Kruijff and Demel.

The structure of the calculated half-channel is shown in Figure 3. In each picture, one molecule of amphotericin B is colored green, while one of sterol is colored yellow.

Figure 3. Calculated structure of an octameric half-channel formed from amphotericin B and cholesterol. (Reproduced by courtesy of Dr. S. Fraga, University of Alberta)

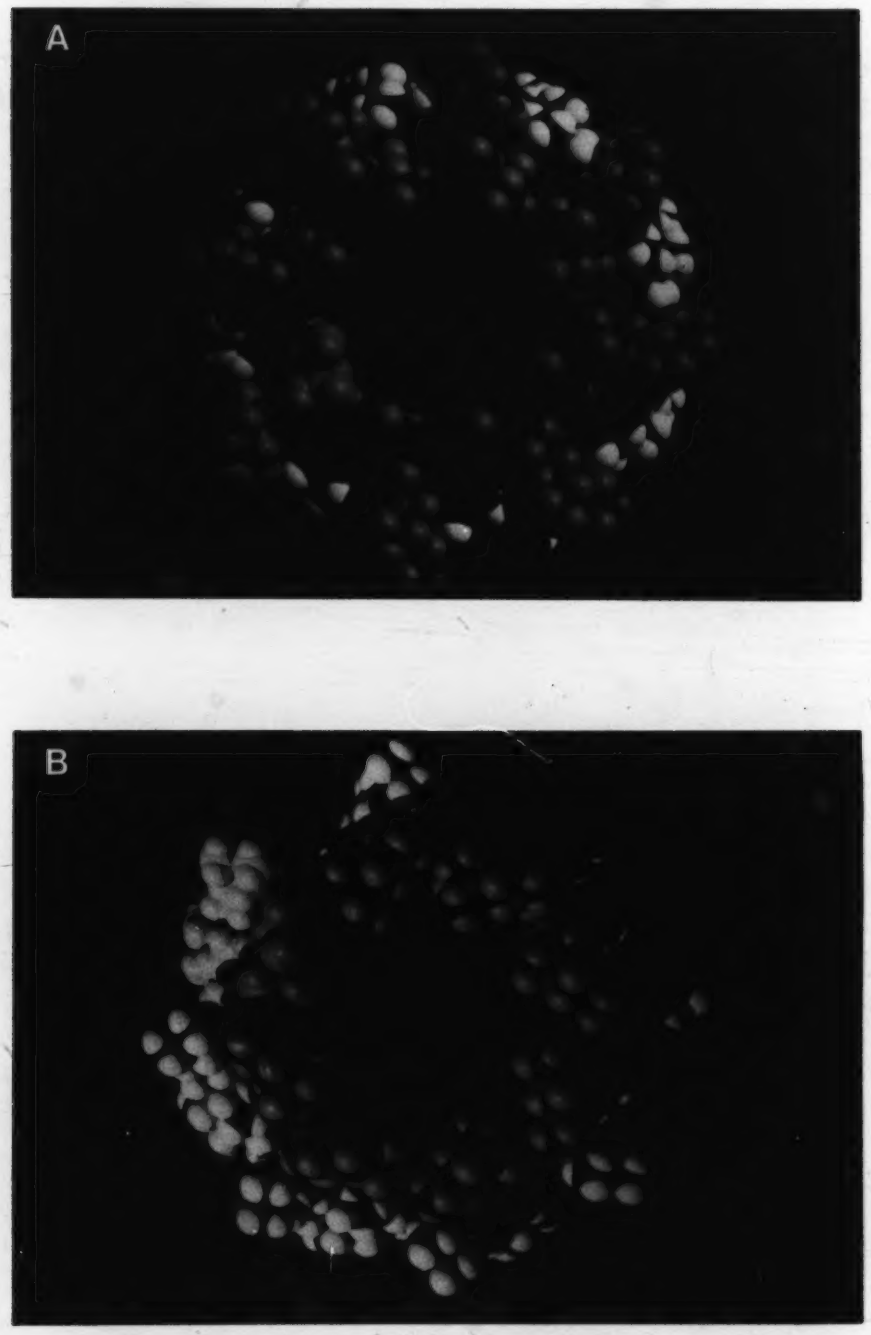
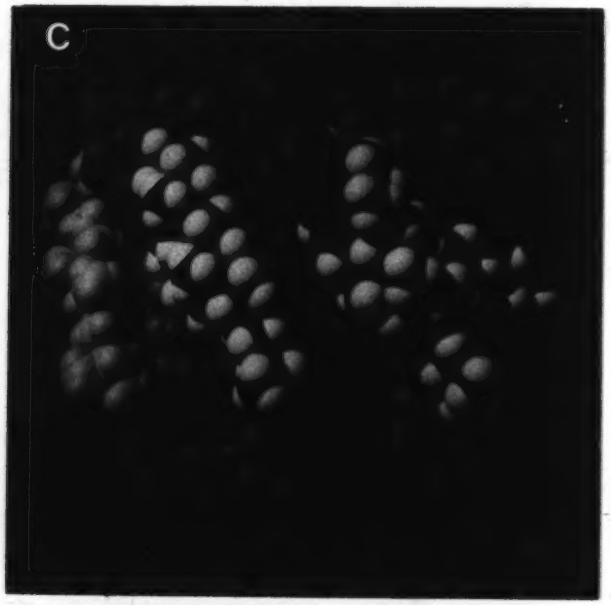


Figure 3 (cont.). Calculated structure of an octameric half-channel formed from amphotericin B and cholesterol. (Reproduced by courtesy of Dr. S. Fraga, University of Alberta)



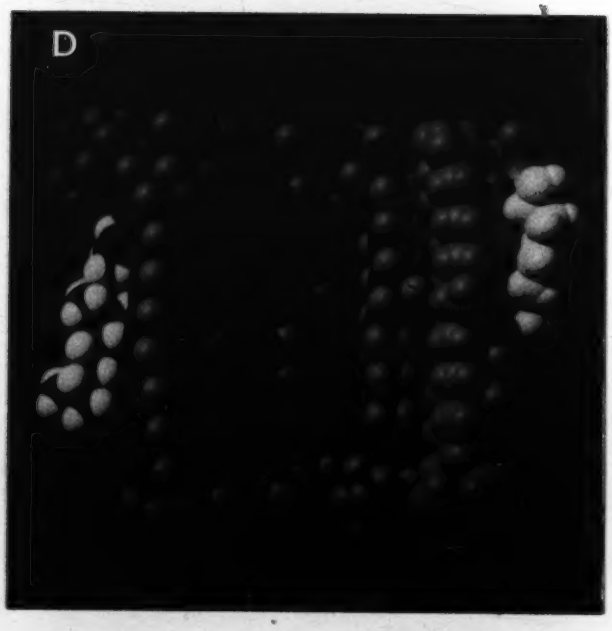


Figure 3a shows the view from above the channel, looking at the hydrophilic surface that is in contact with the aqueous medium. The blue nitrogens of the mycosamine moiety, and a large number of red hydroxyl groups, are clearly evident. A lip of hydroxyl groups around the top of the channel is also apparent. In contrast, Figure 3b shows the view from the other end of the channel. This primarily hydrophobic region would lie at the centre of the membrane, surrounded by lipid. The hydrophobic nature of the outside of the cylindrical channel is clear from Figure 3c, a view from the side. The twisting of each sterol molecule away from the perpendicular is clearly visible. In Figure 3d, the channel has been bisected, permitting a view of the inside. The proximity of the polyene chains to one another is now apparent.

The calculated interaction energies for different sterols may prove useful for comparison with experimentally observed binding selectivities between sterols.

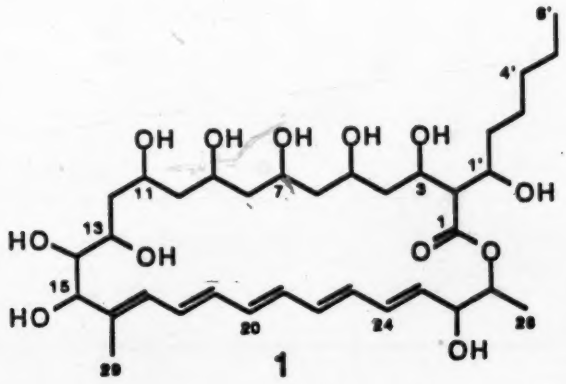
The lack of experimental data on the geometry of interaction between polyenes and sterols shows the need for the development of novel methods of determining this important parameter in the spectrum of variables that control the biological mechanism of action of polyene antibiotics. Knowledge of this geometry could allow logical development of novel polyenes that bind ergosterol (4) more effectively, or that bind cholesterol (3) less, which should reduce the toxicity and increase the effectiveness of these important anti-fungal agents.

II Results and Discussion

Biosynthetic Studies on Fungichromin

One potential approach to detailed investigation of the interaction between polyene antibiotics and sterols uses NMR. The array of NMR methods now available with the advent of high-field, Fourier-transform,[182] and multidimensional NMR[183] makes this technique an extremely powerful tool for structural elucidation.[184] In order to use this technique effectively, complete assignment of the NMR signals is essential. Despite the methods now available, such assignment is often not a straightforward task with complex organic molecules. For example, the assignment of the carbon-13 NMR signals of fungichromin (1) in the region from C-3 to C-12 proves difficult.

The belief that biosynthetic studies could aid in NMR signal assignment, and current interest in biosynthesis,[106, 185, 186] prompt investigation of the biosynthesis of fungichromin (1) using stable isotope labelling techniques. The antibiotic **1** is produced by *Streptomyces cellulosae* .[30, 187] The compound is identical with lagosin[188] from *Streptomyces roseoluteus* and with cogomycin[189] from *Streptomyces fradiae* .[51] The structure has been determined by Cope *et al* .[49] The structure of cogomycin has also been reported.[189, 190]



1

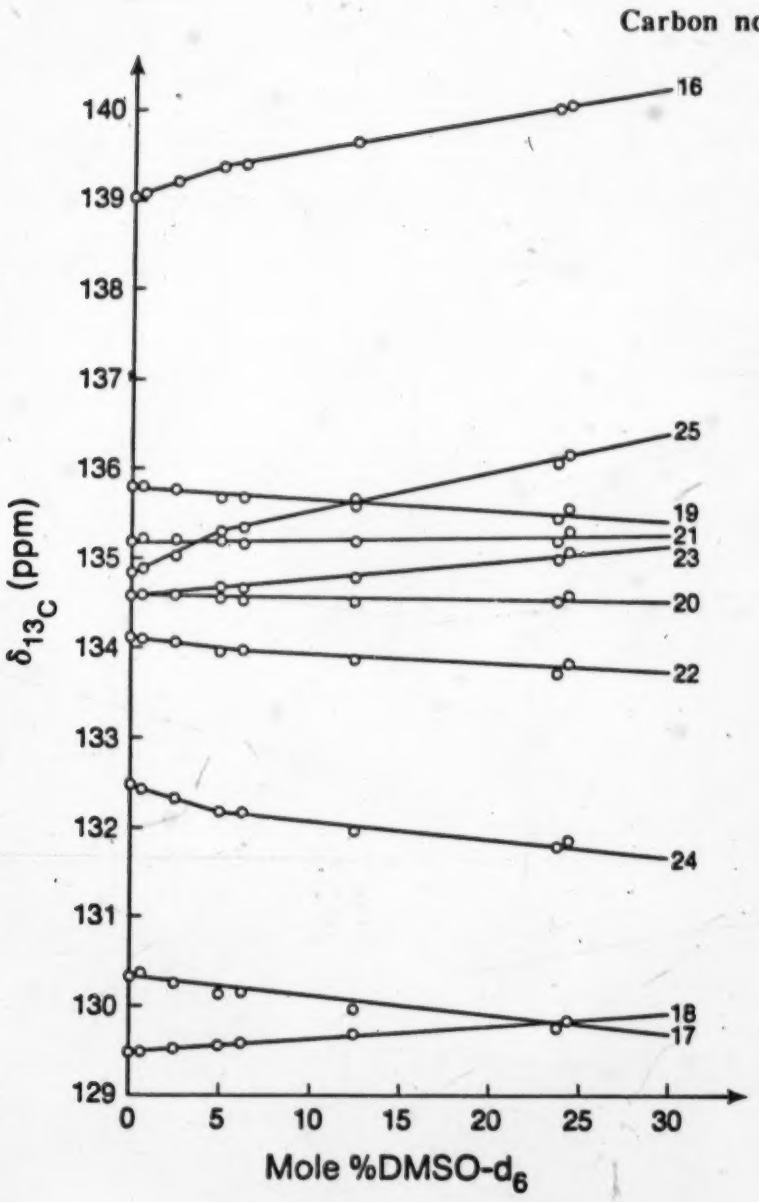
Initial experiments by Dr. H. Noguchi (University of Alberta) established conditions for culturing this organism, and a method for isolation and purification of **1**. Dr. Noguchi also performed the biosynthetic studies with sodium [1-^{14}C]acetate, [1-^{13}C]acetate, [2-^{13}C]acetate, [1-^{13}C]propionate, [1-^{13}C]octanoate, [1-^{13}C]hexanoate, [1-^{13}C, $^{18}O_2$]acetate, [1-^{13}C, $^{18}O_2$]octanoate (**10a**), [2-^{13}C, D_3]acetate, and part of the experiment with D_2O, as well as some of the proton-proton decoupling experiments on **1**.

NMR SIGNAL ASSIGNMENT OF FUNGICHROMIN

Initial NMR experiments using proton[21, 59, 191] and carbon-13[51] chemical shifts, proton-proton decoupling, the proton-proton 2-dimensional COSY spectrum,[44, 45, 192, 193] and proton-carbon-13 heteronuclear shift correlations,[45, 193, 194] allow assignment of many of the proton and carbon-13 signals of fungichromin (**1**).

In order to obtain sufficient **1** in solution for the multidimensional NMR experiments, varying amounts of DMSO-d_6 in methanol-d_4 must be used as solvent. Optimum solubility for **1** is obtained when the DMSO is added before the methanol, thus precluding the use of a standard solvent mixture. Also, many of the early experiments using [1-^{13}C]- or [2-^{13}C]-acetate were performed in methanol alone, since at that point smaller amounts of antibiotic were obtained. Since solvent influences chemical shifts, it must be established whether the carbon signals in **1** change positions as the solvent varies. The partial results of a series of experiments are shown in Figure 4. The mole fraction of DMSO-d_6 is obtained by comparison of the intensities of the carbon signals of the two solvents; the signals are always obtained under identical conditions on the same spectrometer. The values do not therefore represent true compositions, because of differences in relaxation rates of the two solvent signals. This technique does, however, allow direct comparison with archived spectra of samples of **1** that are no longer available, or which have been stripped of solvent. The chemical shifts of some of the polyene carbons

Figure 4. Carbon-13 NMR chemical shifts of the 'polyene' region of fungichromin (1) as a function of DMSO concentration.



clearly cross over or coalesce at certain concentrations of DMSO. An example is shown in Figure 6 (see later), where C-17 and C-18 are at identical chemical shifts. Careful examination of the rest of the ^{13}C spectrum shows that, although chemical shifts do change, no crossover of any of the non-polyene signals occurs with change in mole fraction of DMSO.

The 2-D COSY spectrum of the 'polyene' region ($\delta_H \approx 6.0$ to 6.6) of 1 is shown in Figure 5. Figures 6 - 8 show heteronuclear shift correlations for fungichromin (1): Figure 6 for the 'polyene' region, Figure 7 for the 'hydroxyl' region ($\delta_H \approx 3.1$ to 4.9), and Figure 8 for the 'methyl and methylene' region ($\delta_H \approx 0.9$ to 3.0).

Thus, H-28 appears as a unique methyl doublet: therefore, C-28 is assigned by correlation (Fig. 8). Decoupling of H-28 assigns H-27, and hence C-27 (Fig. 7). Upon decoupling H-26, signals at H-25 and H-27 partially collapse. Thus, C-26 (Fig. 7) and C-25 (Fig. 6) can be assigned. From H-25, the COSY spectrum (Fig. 5) gives H-24, and hence C-24 (Fig. 6). Also, from the COSY spectrum, H-17 may be assigned, since it couples to only one other proton in the polyene region, which is thus H-18. H-18 couples to H-19 (Fig. 5). Assignments for C-17, C-18 and C-19 follow (Fig. 6).

The signals for H-20, H-21, H-22 and H-23 are superimposed, preventing direct signal assignments (Figs. 5 and 6). However, the macrocyclic ring of fungichromin (1) is derived biosynthetically from 12 acetate units and 1 propionate unit, assembled in a "head-to-tail" fashion (see later). Thus, addition of sodium [1,2-$^{13}C_2$]acetate to cultures of *S. cellulosae* during growth leads to incorporation into 1. Further, the units incorporate intact, so that whenever one enriched atom of ^{13}C is present, the adjacent atom within that unit will also be ^{13}C. Coupling between units is not normally observed because the probability of two adjacent units both being enriched is low. The observed ^{13}C signals in 1 are thus doublets, due to coupling between the two ^{13}C atoms, flanking the singlets due to natural abundance ^{13}C signals.

Figure 5. (a) Normal ^{1}H spectrum, and (b) ^{1}H-^{1}H COSY spectrum of the 'polyene' region of fungichromin (1).

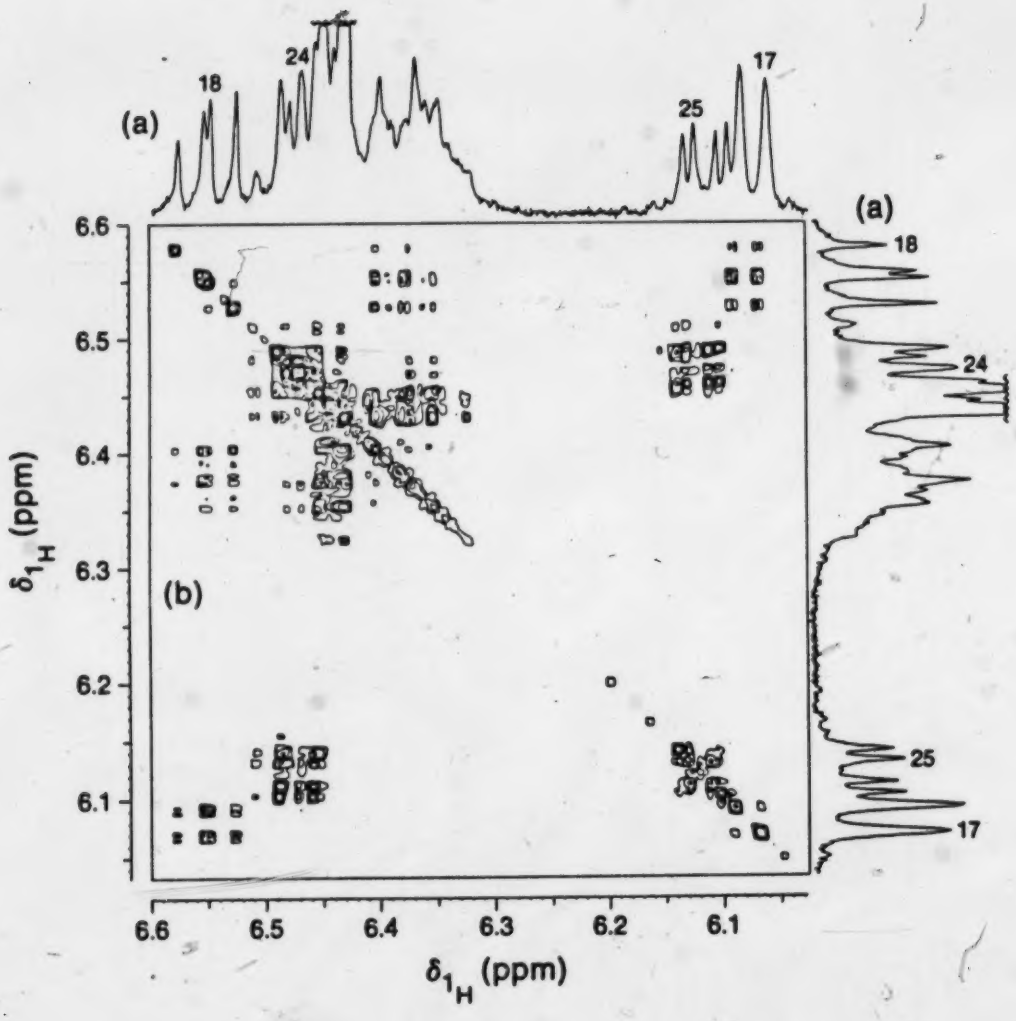


Figure 6. (a) Normal ^{13}C spectrum, (b) normal ^{1}H spectrum and (c) 2-dimensional ^{1}H-^{13}C heteronuclear shift correlation for the 'polyene' region of fungichromin (1).

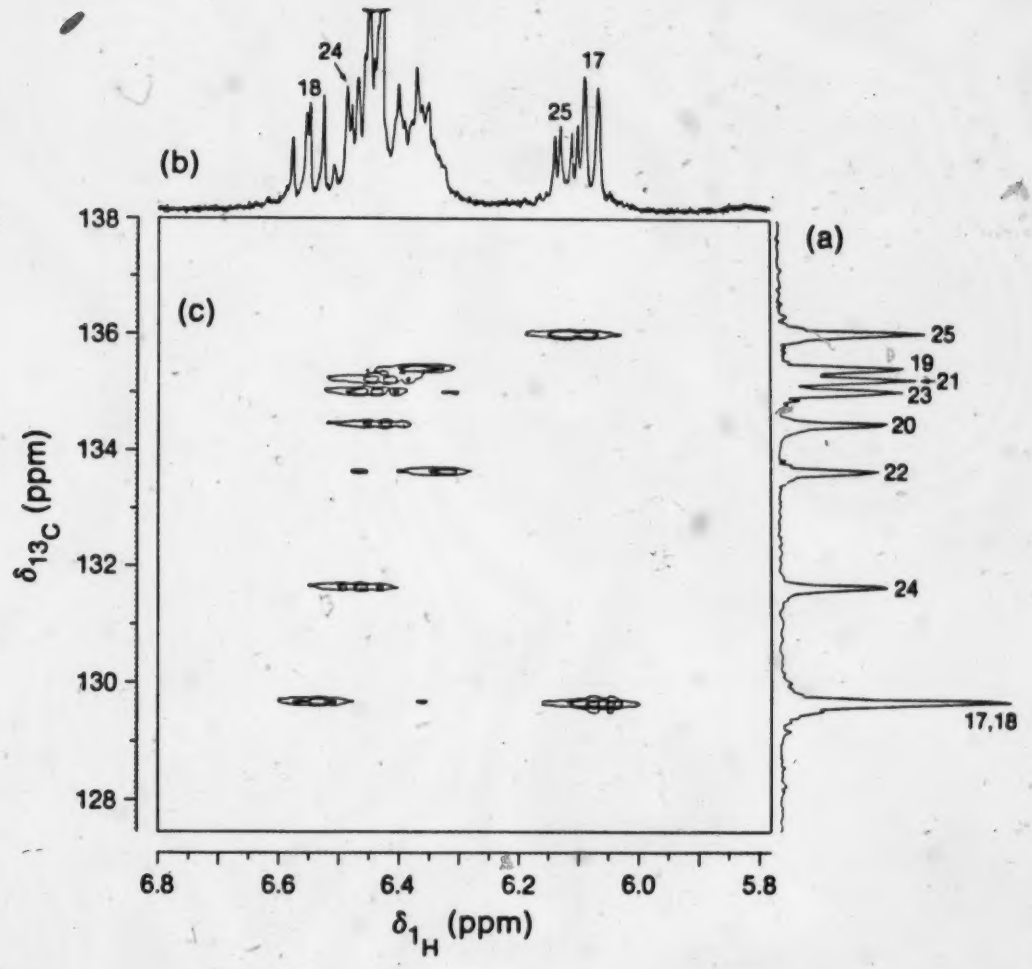


Figure 7. (a) Normal ^{13}C spectrum, (b) normal ^{1}H spectrum and (c) 2-dimensional ^{1}H-^{13}C heteronuclear shift correlation for the 'hydroxyl' region of fungichromin (1). Peaks marked * are folded in.

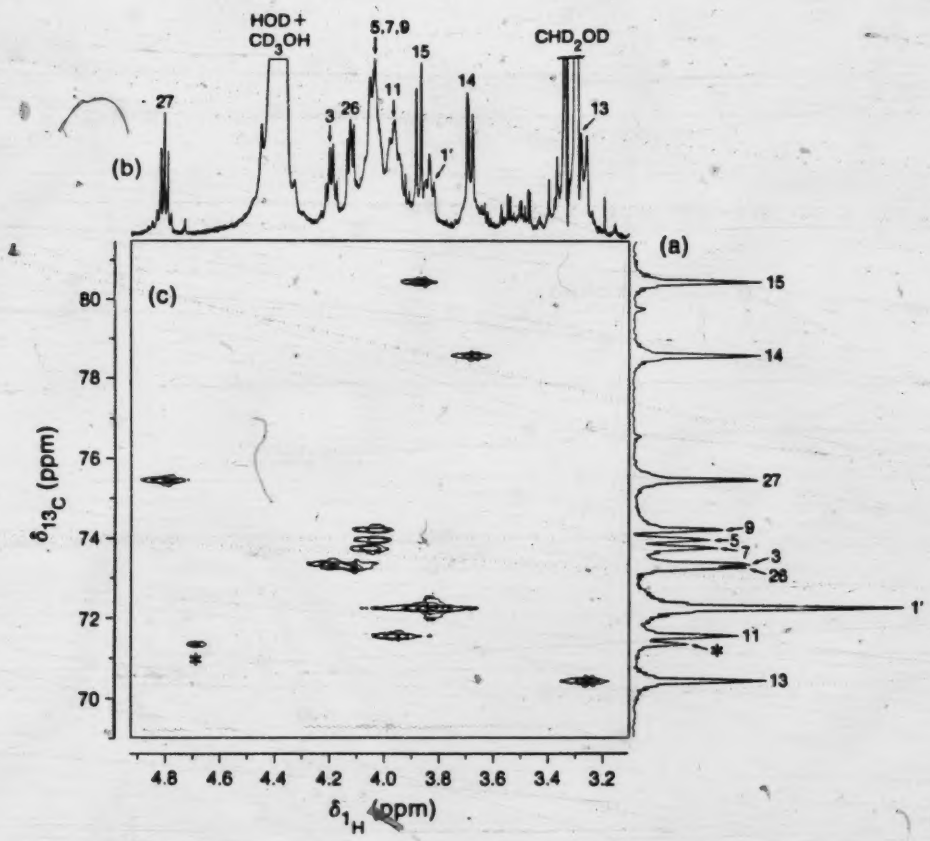
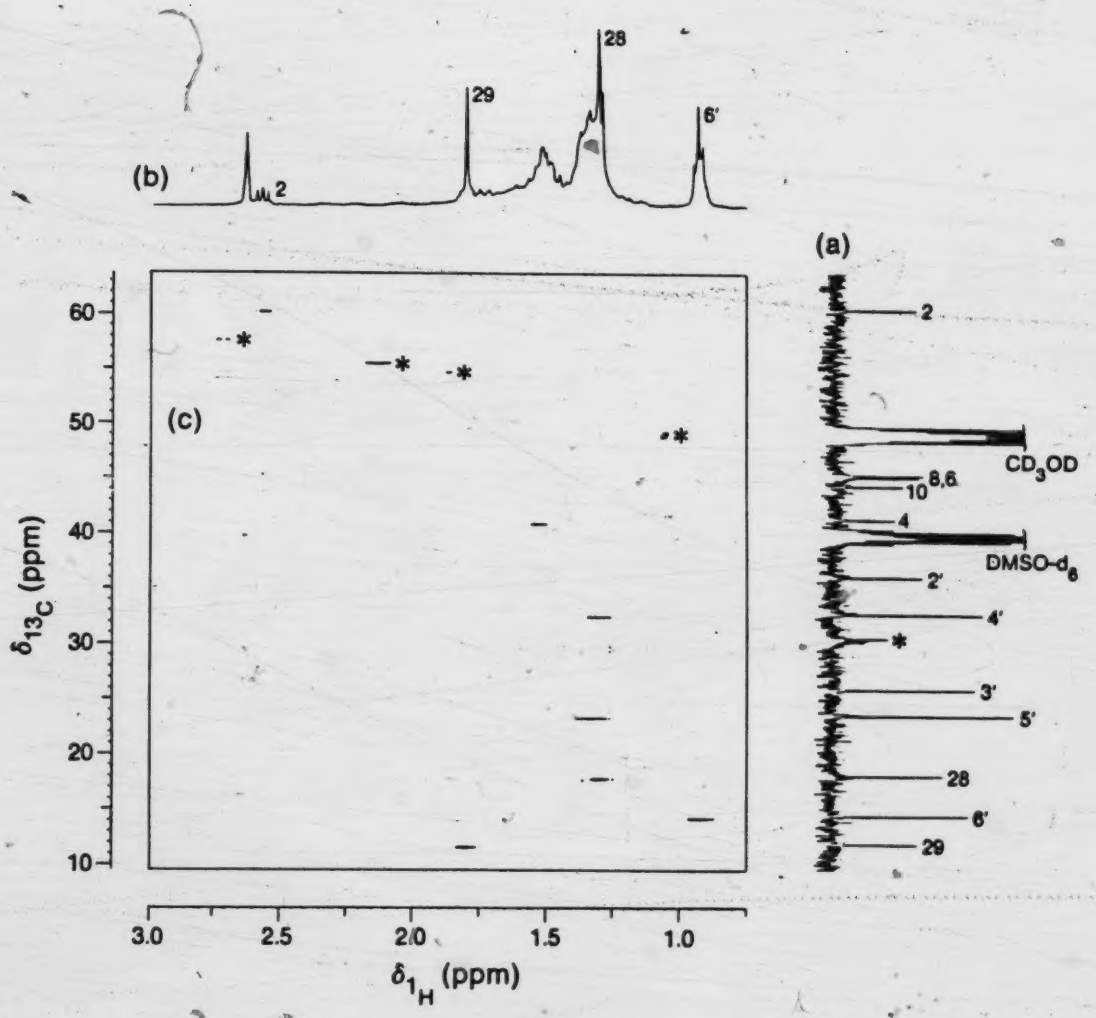


Figure 8. (a) Normal ^{13}C spectrum, (b) normal ^{1}H spectrum and (c) 2-dimensional ^{1}H-^{13}C heteronuclear shift correlation for the 'methyl and methylene' region of fungichromin (1). Peaks marked * are folded in.

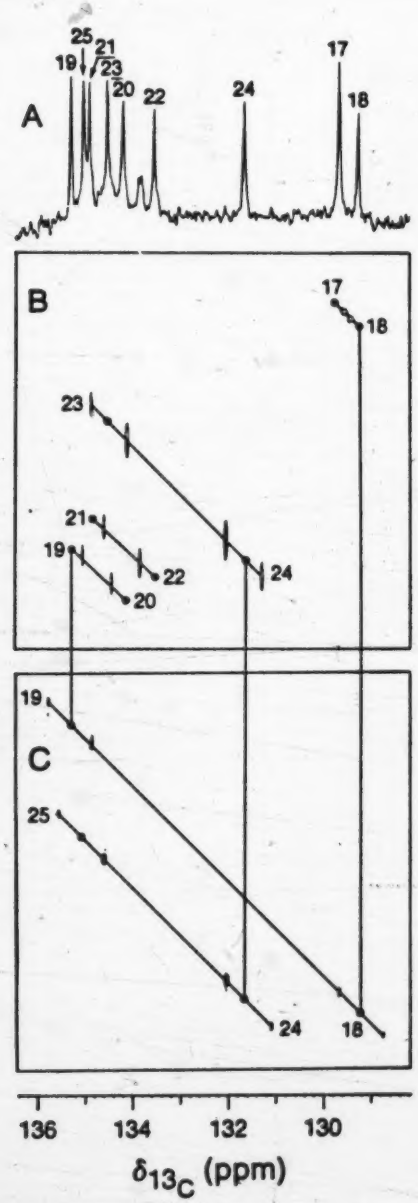


The connectivity between enriched pairs of carbon atoms can now be determined using the two-dimensional INADEQUATE[195] experiment. For the polyene region of 1, the results are shown in Figure 9b. The connectivity between the known pair of carbons, C-17 and C-18, is observed as expected. C-20 is readily assigned by its connectivity to the known C-19, as is C-23 from C-24. The residual signals, due to C-21 and C-22, are also coupled, but this experiment does not distinguish the two. The final assignment is based on the expected "head-to-tail" incorporation of acetate units. This arrangement is found throughout the rest of the molecule, and has extensive precedent in polyketide biosynthesis.[196, 197] Thus, the signal that is enhanced by incorporation of sodium [1-^{13}C]acetate is assumed to be C-21, while that enriched by [2-^{13}C]acetate is C-22. C-16 is readily assigned by an attached proton test,[198] being the only quaternary carbon in the polyene region. Assignment of C-29 is also simple, since H-29 gives rise to a unique methyl singlet (Fig. 8).

Assignment of H-13, H-14 and H-15 is easily accomplished by ^{1}H {^{1}H} selective decoupling experiments. C-13, C-14 and C-15 follow from the shift correlation (Fig. 7). C-1, C-2 and H-2 are assigned by virtue of their unique chemical shifts, and from the shift correlation (Fig. 8). Selective decoupling of H-2 permits assignment of H-1' and H-3, but does not distinguish the two. The corresponding carbon signals, obtained from the shift correlation (Fig. 7), were assigned on biosynthetic evidence: the signal enriched by sodium [1-^{13}C]acetate is assigned to C-3. The other signal is C-1', which is not enriched by acetate (see later). The assignment for C-6' is based on H-6', a unique methyl triplet. Assignments for C-2' to C-5' are those of Pandey,[51] and are based on extensive studies of ^{13}C chemical shifts for compounds with long alkyl chains.[189]

The northern hemisphere (C-3 to C-12) of fungichromin (1) proves to be the most challenging part of the molecule for assignment. In the proton NMR spectrum, H-5, H-7, H-9, and H-11 give rise to closely overlapping multiplets (Fig. 7b), as do the diastereotopic pairs H-4, H-6, H-8, H-10 and H-12 (Fig. 8b). Attempts to resolve these

Figure 9. The 'polyene' region of fungichromin (1): (a) normal ^{13}C spectrum, (b) 2-dimensional tilted INADEQUATE spectrum of 1 enriched by incorporation of [1,2-$^{13}C_2$]acetate and (c) 2-dimensional tilted INADEQUATE spectrum of 1 enriched by incorporation of a mixture of diethyl [2-^{13}C]- and [1,3-$^{13}C_2$]-malonates.



signals by using different solvents, and by using various metal ions in the hope of obtaining selective chelation of certain hydroxyl groups, were unsuccessful. It was hoped that transannular nuclear Overhauser effects (n.O.e.'s) could be observed between the known protons in the polyene chain and the unknown protons in the polyol moiety. However, attempts to observe either one- or two-dimensional n.O.e.'s were unpromising. The absence of n.O.e. may be due to the molecular weight (670) of **1**, which is close to that at which theory predicts absence of the effect.[199, 200] The long-range proton-to-carbon relayed correlation experiment[201] merely furnishes extra evidence for some of the assignments already made; it gives no new information, perhaps because of the fast relaxation of the carbon atoms in the northern hemisphere of **1** manifested in the observable line broadening of these signals. The relayed correlation experiment requires a long delay between preparation and detection. Therefore, the magnetisation of interest has decayed to a large extent by the time aquisition starts.

Thus, NMR techniques involving protons appear not to be of help in this problem. However, the larger chemical shift dispersion available in the carbon-13 spectrum makes this ideally suited to assignment. The normal techniques used in this approach are one-[183] or two-dimensional INADEQUATE,[183, 195] or ^{13}C-^{13}C COSY.[202] These experiments have been used extensively in structural elucidation and assignment,[184] and in biosynthetic studies.[203] However, the low natural abundance of ^{13}C (1.1%) makes detection of coupled signals (≈1% of 1%) difficult; thus, large amounts of material are required. The biosynthetic approach involving incorporation of doubly-labelled precursors that are incorporated intact into a natural product (e. g. sodium [1,2-$^{13}C_2$]acetate into fungichromin (**1**)) allows *some* of the carbon-carbon connectivities to be determined. However, the full carbon-carbon connectivity pattern cannot normally be obtained in this way. Much smaller amounts of material are required in these cases. For example, the spectrum described above (Fig. 9b) was obtained on 25 mg of **1**, with an incorporation from acetate of ca 1%. Even if sufficient **1** were available for a natural abundance

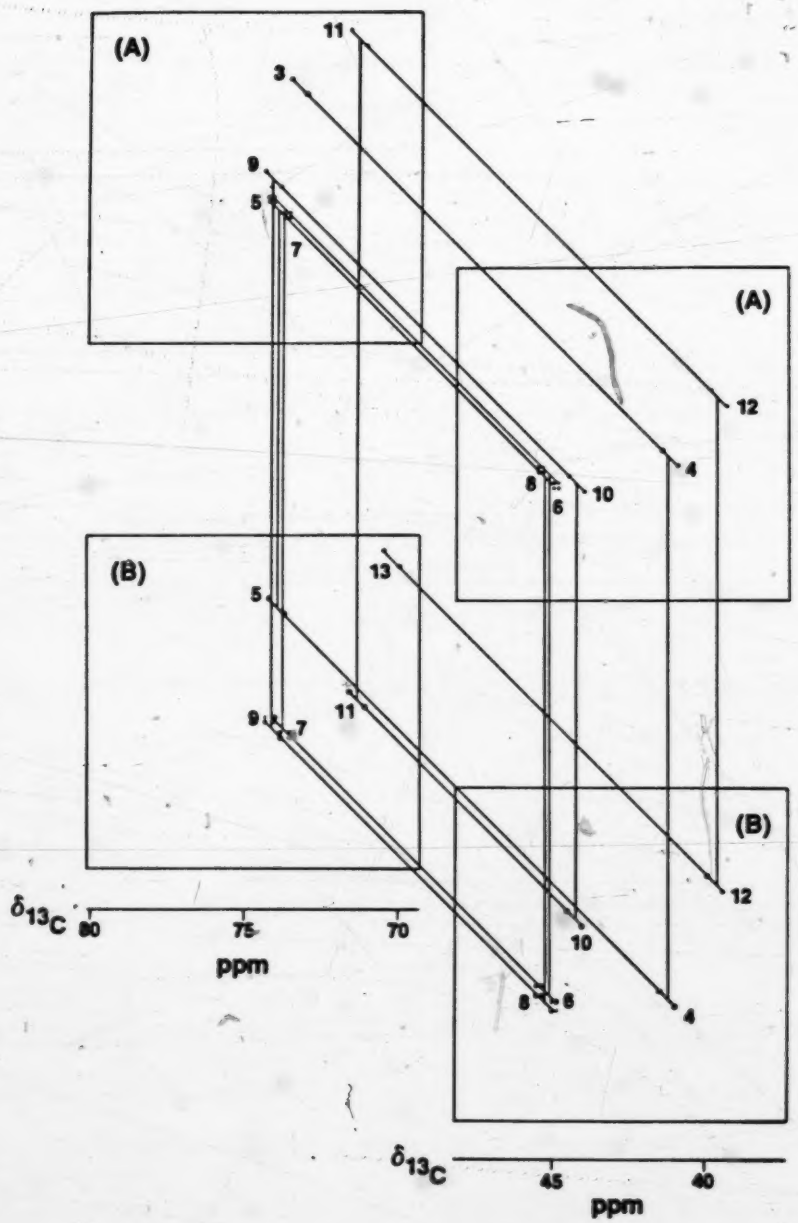
INADEQUATE spectrum using available spectrometers, it could not be dissolved in a 5 or 10 mm NMR tube.

Parts of the two-dimensional INADEQUATE spectrum of the hydroxyl and methylene region ($\delta_C \approx 35$ to 82) of fungichromin (1) enriched by sodium [1,2-$^{13}C_2$]acetate are shown in Figure 10a. Each set of signals in the two boxes is a section expanded from the full spectrum for clarity, with the empty area between $\delta_C \approx 48$ to 69 removed. With C-3 assigned as described above, C-4 is easily assigned. Correlations also are clear between other pairs of signals. However, absolute assignments cannot be made due to the lack of coupling information between adjoining units. For example, the signal labelled as C-11 is linked to that of C-12. However, at this point, neither signal has been assigned by other techniques, and thus this correlation could be that between any of the pairs C-5 and C-6, C-7 and C-8, C-9 and C-10, or C-11 and C-12.

However, if a precursor could be found that labelled each acetate-derived site more efficiently than the 1% incorporation for acetate itself, couplings between units might be observable. The full carbon signal assignment of the northern hemisphere of 1 could then be determined.

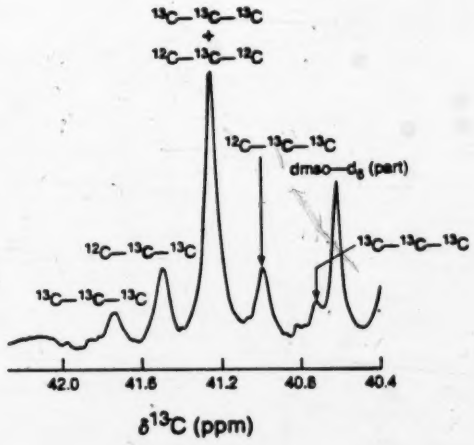
The first approach to this labelling problem used glucose. A trial experiment in which [U-^{14}C]glucose is fed gives radioactive 1. If all the measured incorporated radioactivity is present in the acetate units, then 40% of the carbon atoms in these units come from glucose. When [U-^{13}C]glucose now totally replaces the normal glucose in the fermentation, the derived fungichromin (1) should have 40% ^{13}C/carbon atom. All the labelled sites will be coupled (intra-unit coupling), since degradation of glucose to acetate occurs with carbon-carbon bonds remaining intact.[204] However, in 40% X 40% of cases, i.e. 16%, two adjacent units will both be labelled, and any given carbon atom will be a ^{13}C with two adjacent ^{13}C atoms. Thus, coupling information between acetate units will be obtained (inter-unit coupling). In practice, the cost associated with this experiment compels use of only 1 gram of labelled glucose, enough for 1 fermentation flask, or ca. 2 mg of

Figure 10. The 'hydroxyl and methylene' region of fungichromin (1): (a) 2-dimensional tilted INADEQUATE spectrum of 1 enriched by incorporation of [1,2-$^{13}C_2$]acetate and (b) 2-dimensional tilted INADEQUATE spectrum of 1 enriched by incorporation of a mixture of diethyl [2-^{13}C]- and [1,3-$^{13}C_2$]-malonates.



labelled 1. The product is therefore isolated using unlabelled carrier material. A typical ^{13}C NMR signal is shown in Figure 11. The central peak is due to natural abundance ^{13}C in unlabelled material from the carrier. This peak is flanked by a doublet, and by the outside wings of a triplet. The doublet is due to ^{13}C from labelled glucose, which is coupled within the acetate unit, but not coupled by the adjacent unit. The triplet, centred at the unenriched peak, is due to the species' containing two acetate units in a row both derived from labelled glucose, and contains the inter-unit coupling information.

Figure 11. Signal due to C-4 of fungichromin (1) enriched from [U-^{13}C]glucose.



When the INADEQUATE experiment is performed on this sample, however, only the doublet signals from intra-unit coupling show correlations. At first sight, this observation appears to be due to the lower probability of inter-unit coupling. However, the intra-unit couplings are intense enough to suggest that the inter-unit couplings should be observed. Theoretical considerations by Dr. T. T. Nakashima and Dr. R. E. D. McClung (University of Alberta) suggest that the double-quantum filter that removes uncoupled singlets in the INADEQUATE experiment may also filter out the desired triplets. Attempts at one-dimensional ^{13}C {^{13}C} homonuclear decoupling[205, 206] were thwarted by the

considerable reduction in signal-to-noise inherent in such experiments. The triple quantum coherence experiment,[207] which is ideal for a system of three coupled spins, was considered impractical, since the signal-to-noise level is expected to be considerably reduced relative to the double quantum experiment.

These results suggest that a precursor that incorporates specifically and with high efficiency as singly-labelled acetate into **1**, is desirable. During the course of other experiments (see later), diethyl [2-^{13}C]malonate was found to incorporate to the 8 - 9% level into all the acetate units, in marked contrast to the reported poor incorporation of diethyl [1,2,3-$^{13}C_3$]malonate into viridicatumtoxin by *Penicillium expansum* .[208] Thus, incorporation of a mixture of diethyl [2-^{13}C]malonate and diethyl [1,3-$^{13}C_2$]malonate (**11a**) gives rise to labelled **1** with ca. 4 - 5% ^{13}C per site. Diethyl malonate (**11b**) is readily available from esterification[209] of the acid, and **11a** was prepared similarly. The enriched signals in the derived **1** are flanked by small doublets, since in a few molecules, one [1-^{13}C]- and one [2-^{13}C]-unit link "head-to-tail" to form a new ^{13}C-^{13}C bond. In this experiment, these inter-unit couplings are the only one-bond ^{13}C-^{13}C couplings. The INADEQUATE spectrum of the hydroxyl and methylene region of **1** labelled in this way is shown in Figure 10b. It contains all, and only, the complementary connectivities to those in Figure 10a. Thus, starting at the known position, C-3, in Figure 10a, the sloping line shows the correlation to C-4. Dropping vertically to the chemical shift of C-4 in Figure 10b, then following the diagonal, shows the correlation of C-4 to C-5. Ascending back into Figure 10a at the chemical shift of C-5 reveals correlation to C-6. Eventually, the connectivity follows round to terminate at the known signal due to C-13. When the full spectrum is examined, connectivity continues from C-13 to C-14, and a confirmatory correlation between C-27 and C-28 is also observed. The connectivity stops at C-2, C-15, and C-16, since these sites are not enriched by acetate (see later).

The results of this novel technique prompted aquisition of the INADEQUATE spectrum of the polyene region of the same sample (Figure 9c), which, combined with the

dilabelled acetate results (Figure 9b), make the connectivity sequences C-17 to 18 to 19 to 20, and C-23 to 24 to 25 readily apparent. However, the expected connectivities C-20 to 21, and C-22 to 23 are absent from Figure 9c. Also, the outer line of each doublet in both spectra is weaker than the inner line. In the polyene region, the chemical shift difference between coupled carbon atoms, $\Delta\delta$, is of the order of, or in some cases less than, the ^{13}C-^{13}C coupling constants, J. This leads to AB patterns in the spectrum, thereby explaining the weaker outer lines. In collaboration with Dr. R. E. D. McClung and Dr. T. T. Nakashima (University of Alberta), theoretical investigations of the influence of these strongly-coupled systems on the signal intensities in the INADEQUATE experiment are in progress. Preliminary results suggest that these intensities fall off rapidly as $\Delta\delta$ decreases towards and then becomes less than J. The experimental intensities within a given pair of coupled doublets agree well with the theoretical calculations. The intensity diminution of strongly-coupled ^{13}C-^{13}C systems has also been observed in both J-resolved[210] and INADEQUATE[211, 212] experiments.

This new technique should prove useful in NMR assignment problems, especially for molecules such as **1** which contain multiple repeating units that are nevertheless chemically inequivalent. A noteworthy feature of this technique is the enhanced signal intensity over and above that expected on purely statistical grounds (ie. ca. 5% X 5%). This enhancement is probably due to the pulsed feeding of diethyl malonate, which may give rise to very high incorporations at adjacent sites just after its addition to the culture. The technique has already been used with conspicuous success in incorporation of a mixture of [1-^{13}C]- and [2-^{13}C]-acetates, as well as of [1,2-$^{13}C_2$]acetate, into dehydrocurvularin. The resulting two INADEQUATE spectra allowed complete assignment of this polyketide metabolite.[213]

With all the carbon atoms of fungichromin assigned, the exact proton chemical shifts within the overlapping multiplets may be obtained from the shift correlations (Figs. 6 - 8). The complete carbon and proton assignment of fungichromin (**1**) is given in Table 1.

Table 1

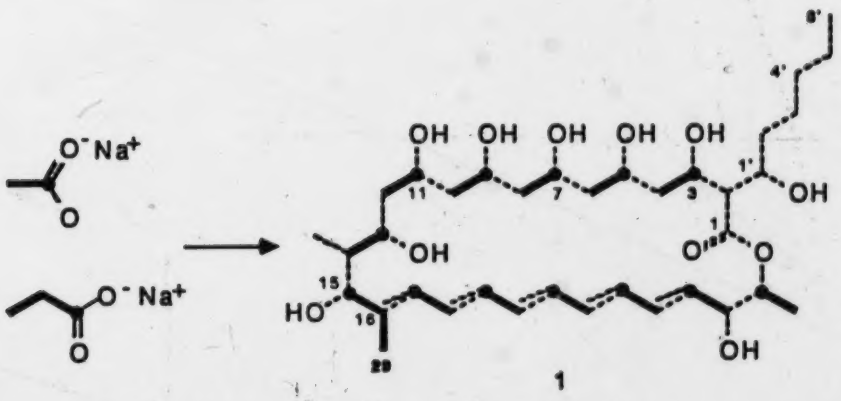
^{13}C and ^{1}H chemical shifts and isotopic incorporations for fungichromin (1)

Carbon[a]	^{13}C δ (ppm)[a]	^{1}H δ[b] (mult., J)[c]	Enhancement(s)[d]	Precursor(s)
1	172.98		2.9; 4.0; 2.8	e; l; m
16	138.55			
19	135.36	6.38 (ddm, 14.4, 11.4)	2.7	f
21	134.81	6.42 (dd, 12, 12)	2.4	f
25	134.28	6.12 (dd, 14.3, 5.0)	2.9	f
23	134.21	6.44 (dd, 12, 12)	2.5	f
20	134.13	6.43 (dd, 12, 12)	2.2; 8	g; k
22	133.66	6.32 (m)	2.5; 8	g; k
24	131.97	6.48 (dm, 14.3)	2.6; 8	g; k
17	129.91	6.08 (dd, 11.2, 2.0)	2.5	f
18	129.06	6.55 (dd, 14.1, 11.2)	2.2; 8	g; k
15	80.43	3.86 (d, 9)	5.6	h
14	78.31	3.55 (dd, 9, 1.5)	2.8; 9	g; k
27	75.25	4.79 (q, 7, d, 7)	2.5	f
9	74.20	4.02 (≈dd, ≈6, ≈6)	2.5	f
5	74.08	4.02 (m)	2.6	f
7	73.92	4.14 (≈p, ≈6)	2.7	f
3	73.41	4.05 (m)	2.3	f
26	73.25	4.07 (dd, 6, 6)	2.7; 9	g; k
1'	72.59	3.70 (m)	2.5	i
11	71.45	3.95 (m)	2.3	f
13	70.34	3.30 (d, 11)	2.5	f
2	60.35	2.55 (dd, 9, 7)		
8	45.33	1.33 (m), 1.49 (m)	2.9; 9	g; k
6	45.17	1.38 (m), 1.47 (m)	2.8; 9	g; k
10	44.34	1.51 (m)	2.5; 9	g; k
4	41.38	1.52 (m)	2.9; 9	g; k
12	39.58	1.39 (m), 1.52 (m)	2.5; 9	g; k
2'	36.22	1.35 (m), 1.52 (m)		
4'	32.88	1.30 (m)		
3'	26.01	1.40 (m), 1.53 (m)		
5'	23.65	1.33 (m)		
28	17.96	1.16 (d, 6)	3.0; 9	g; k
6'	14.38	0.88 (t, 7)	110±20	j
29	11.74	1.78 (s)	50±10	j

[a]100.6 MHz ^{13}C NMR spectrum in methanol-d_4 with solvent reference at 49.00 ppm. [b]400 or 500 MHz ^{1}H NMR spectrum in 25 mol % DMSO-d_6/methanol-d_4 (see text), relative to internal TMS at 0.00 ppm. [c]Multiplicities (mult.) and coupling constants (J, in Hz) determined from the normal ^{1}H spectrum, the 2D-COSY spectrum or slices through the ^{13}C-^{1}H heteronuclear shift correlation. [d]Ratio of carbon signal intensities for enriched and natural abundance samples measured under identical conditions. [e]Sodium [1-^{13}C]octanoate. [f]Sodium [1-^{13}C]acetate. [g]Sodium [2-^{13}C]acetate. [h]Sodium [1-^{13}C]propionate. [i]Sodium [3-^{13}C]octanoate (**10b**). [j]Ethyl [CD_3]oleate (**18a**). [k]Diethyl [2-^{13}C]malonate. [l]S-([1-^{13}C]Octanoyl)-N-acetylcysteamine (**17b**). [m]Ethyl [1-^{13}C]octanoate (**16b**).

With these results the biosynthetic source of the carbon atoms in fungichromin (1) can be determined. A preliminary experiment with sodium [1-^{14}C]acetate shows encouraging incorporation into 1. Addition of sodium [1-^{13}C]acetate to cultures of *S. cellulosae* during growth gives 1 which is enriched at twelve alternating positions around the macrocyclic ring (Figure 12c). A similar experiment with sodium [2-^{13}C]acetate gives the complementary result (Figure 12d). Incorporation levels are given in Table 1. Incorporation of sodium [1,2-$^{13}C_2$]acetate gives rise to coupled signals (see above and Figures 9b and 10a), indicating that each of the acetate units is incorporated intact (Scheme 1). Incorporation of sodium [1-^{13}C]propionate gives 1 labelled specifically at C-15 (Figure 12b), which indicates that the three-carbon fragment, C-15, C-16 and C-29, is derived from one propionate unit.

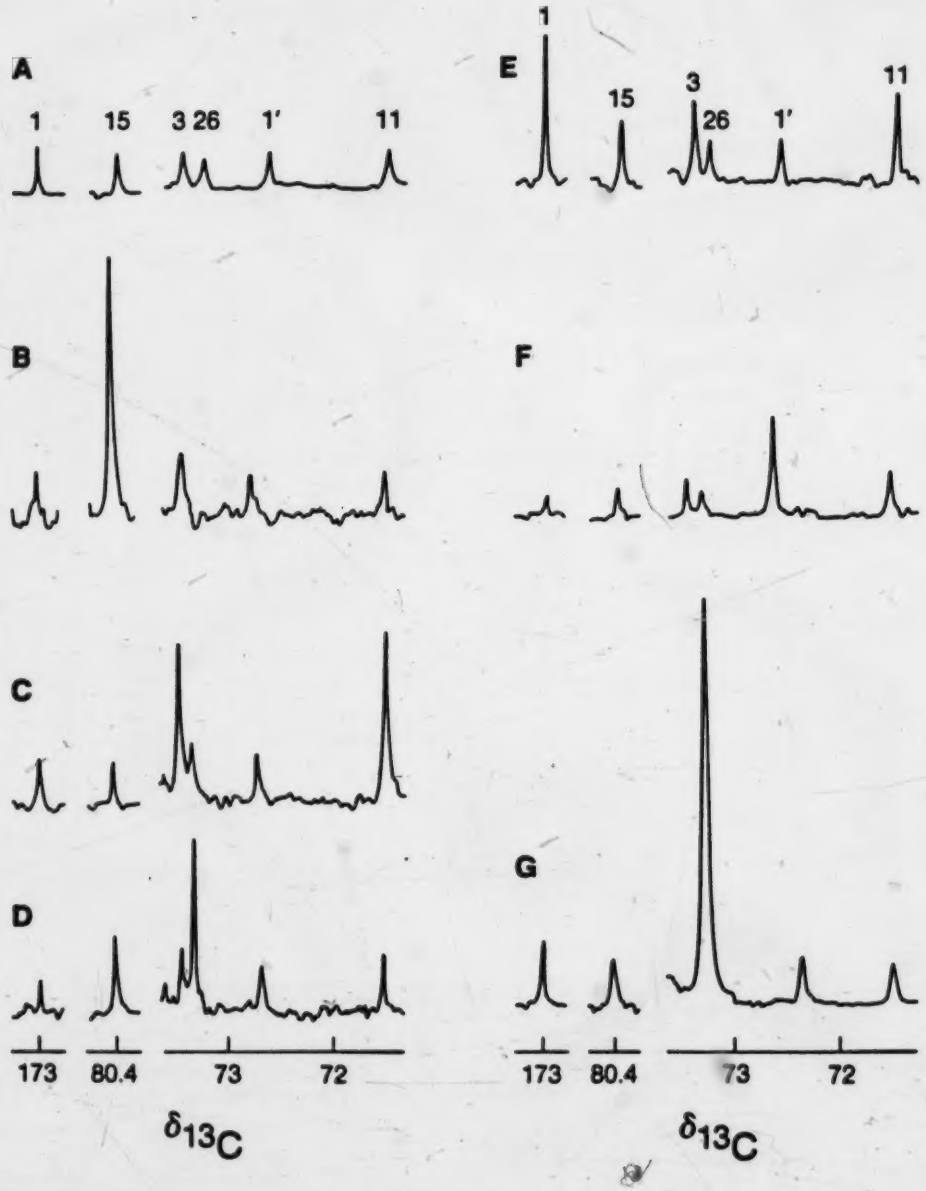
Scheme 1.



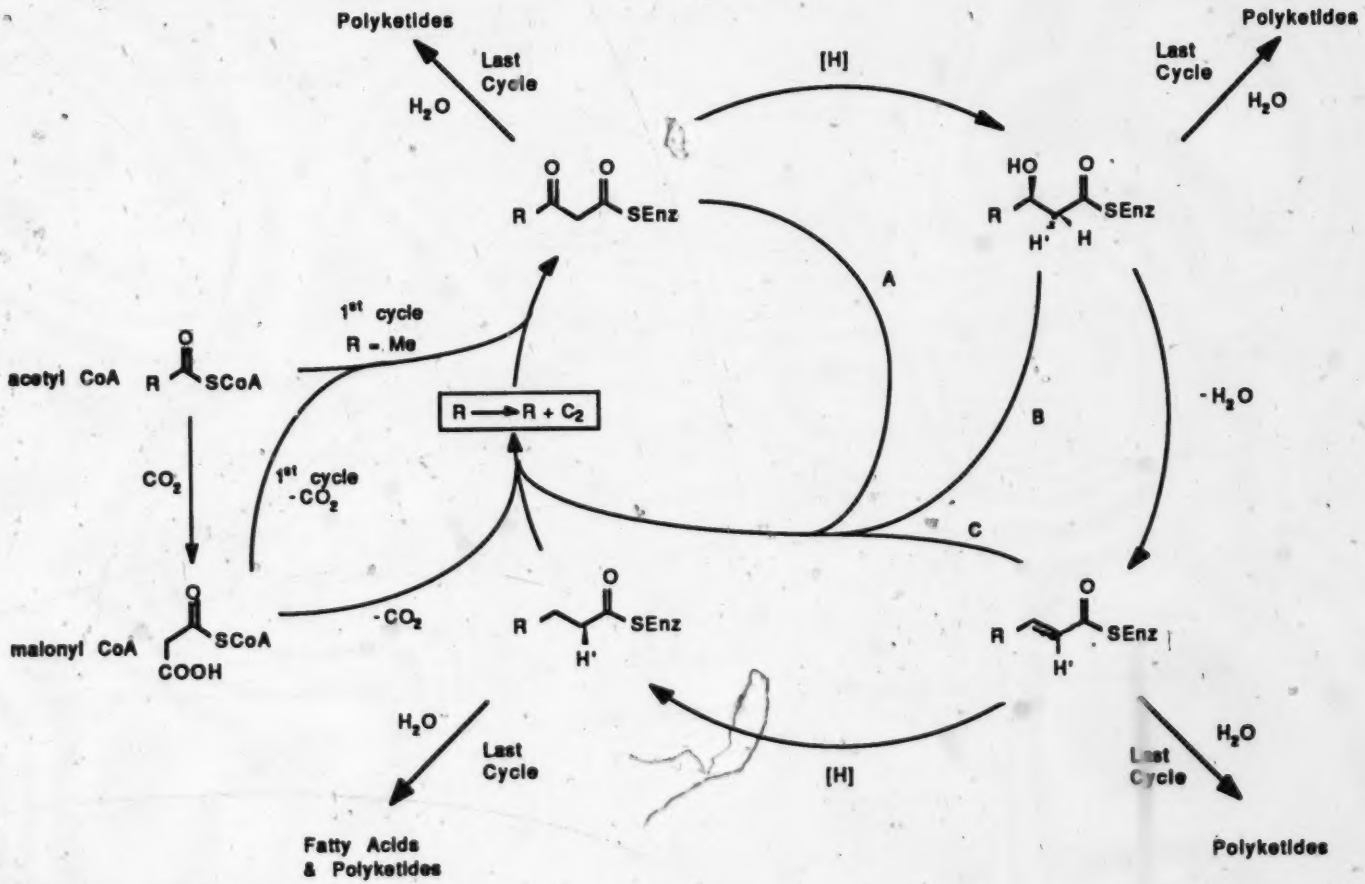
1

These results agree with normal polyketide biosynthesis (Scheme 2).[106] The Birch-Collie hypothesis postulates that polyketide secondary metabolites derive from head-to-tail coupling of acetate or propionate units.[196, 197] Occasionally, butyrate is incorporated intact, as in the biosynthesis of ebelactone B[214] and leptomycin B[215] by

Figure 12. Partial ^{13}C NMR spectra of fungichromin (1): (a) natural abundance, and (b) to (g), enriched by incorporation of: (b) sodium [1-^{13}C]propionate, (c) sodium [1-^{13}C]acetate, (d) sodium [2-^{13}C]acetate, (e) sodium [1-^{13}C]octanoate, (f) sodium [3-^{13}C]octanoate, and (g) diethyl [2-^{13}C]malonate.



Scheme 2. Fatty acid and polyketide biosynthesis.[106]



Streptomyces spp.. The starter acetate unit, bound to the enzyme as a thioester, condenses with enzyme-bound malonate to form a C_4 enzyme-bound β-ketoester. This ester may undergo reduction to the corresponding β-hydroxyester. Elimination of water then gives the α,β-unsaturated material, which is reduced to the saturated enzyme-bound butyrate. The butyrate unit may then undergo repeated condensations with malonate, adding two carbons at a time, and continuing around the cycle. Alternatively, a similar condensation with methylmalonate introduces three-carbon (propionate) units into the chain. In fatty acid biosynthesis, this process usually stops at the C-16 to C-18 stage: hydrolysis then gives the free fatty acid. In polyketide biosynthesis, the reduction-elimination-reduction process does not necessarily go to completion at each stage, resulting in long carbon chains that have carbonyl, hydroxyl, or double bond moieties along the chain, depending on whether paths A, B or C in Scheme 2 are followed. Polyketide metabolites such as fungichromin (1) result from the subsequent or *in situ* reactions of these species.

In contrast to the 2- to 3-fold enhancements observed in the macrocyclic ring of 1 upon addition of labelled acetate or propionate, detectable enhancements in the signals due to C-1, C-2 or C-1' to C-6' were not observed in these experiments (Figure 12b, c, d). Indeed, no coupled satellites are observed around any of these signals in the experiment with [1,2-$^{13}C_2$]acetate, which has been shown to be more sensitive to small incorporations of labelled precursors.[216, 217] Thus, small coupled signals are observed around C-29, due to conversion of acetate to propionate via succinyl-CoA and the citric acid cycle.[203]

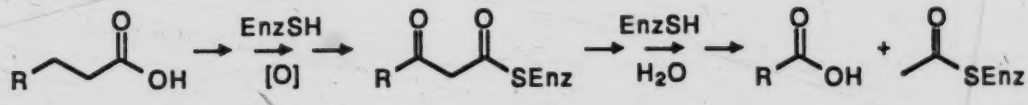
Martin,[218] on the basis of incorporation experiments with radioactive acetate,[87] concludes that the structurally similar polyene, filipin (2),[29, 219] from *Streptomyces filipinensis*, is a polyketide metabolite. Filipin (2) co-occurs with fungichromin (1) in isolates from *S. cellulosae*. It thus appears that the biosynthesis of these two compounds is closely related, probably with oxidation of 2 to 1 occurring close to the last step. For the biosynthesis of filipin, Martin implies the possible intermediacy of octanoate in formation of the C-1 to C-6' fragment. The octanoate is suggested to derive from condensation of

four acetate units.

The results with fungichromin (1) clearly do not support this hypothesis. However, octanoate derived from a source other than acetate may be a precursor. Upon incorporation of sodium [1-^{13}C]octanoate into 1, specific enhancement of C-1 is observed (Figure 12e and Table 1). Only small (ca. 25%) enhancements of the acetate-derived positions, due to degradation of octanoate, occur.

These results show an unprecedented direct incorporation of a long-chain fatty acid with more than 4 carbon atoms, as an intact unit, into a polyketide, without extensive β-oxidation to acetate. β-Oxidation is normally rapid,[220] and most attempts to incorporate labelled fatty acids result in incorporation of label from acetate only after degradation has occurred (Scheme 3). In the case of averufin biosynthesis, Townsend *et al* [221] observed intact incorporation of [1-^{13}C]hexanoate, but also considerable incorporation from [1-^{13}C]acetate derived by β-oxidation.

Scheme 3.



Feeding sodium [1-^{13}C]hexanoate to cultures of *S. cellulosae* , to test the specificity of octanoate as a unit, gives 1 in which none of the carbon atoms are labelled. This suggests that hexanoate is neither incorporated direct or via acetate into 1, and further, that β-oxidation of hexanoate in this organism is slow, since no degradation to acetate apparently occurs.

This unusual result merits independent confirmation. Thus, a synthesis of sodium [3-^{13}C]octanoate (10b) was sought. Retrosynthetic analysis, with the requirement of easy incorporation of the label in mind, suggests an approach from hexanoic acid. Diborane

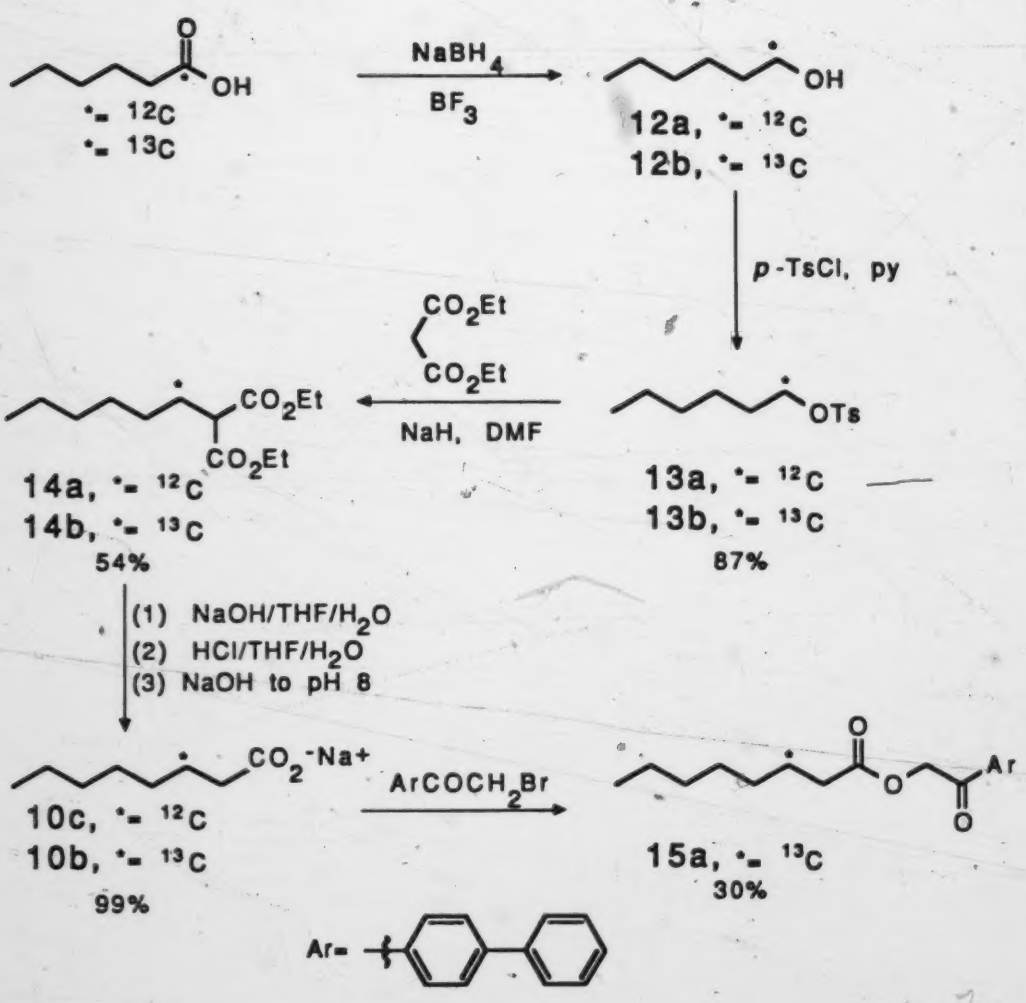
reduction of hexanoic acid,[222] although slow, affords hexanol (**12a**), which, without isolation, reacts with *p* -toluenesulfonyl chloride in pyridine to give the isolable hexyl *p* -toluenesulfonate (**13a**)[223] (Scheme 4). Displacement with the anion of diethyl malonate[224] gives diethyl hexylmalonate (**14a**),[225] in modest yield. Schwab *et al* [223] have more recently reported an improved procedure for this step. Hydrolysis of **14a** in base to the di-sodium salt which decarboxylates upon acidification, gives octanoic acid,[225] isolated as the sodium salt **10c**. The procedure can be used on [1-^{13}C]hexanoic acid to give sodium [3-^{13}C]octanoate (**10b**).

In order to fully characterise **10b**, and to establish the extent of isotopic labelling by mass spectrometry, the material must be derivatised. Compound **10b** reacts with *p*-phenylphenacyl bromide using the method of Risley and Van Etten[226] to give *p* -phenylphenacyl ester **15a** (Scheme 4). The mass spectrum of **15a** shows no detectable molecular ion from unenriched material, indicating the isotopic purity to be ≥ 99% ^{13}C.

Sodium [3-^{13}C]octanoate (**10b**) incorporates efficiently into fungichromin (**1**). The ^{13}C NMR spectrum shows C-1' to be the sole site of enrichment as expected (Figure 12f and Scheme 5). In this case, there is no detectable incorporation of label as acetate, which would be expected if 2 or more sequential β-oxidations occur. The two results clearly show that octanoate is a preferred precursor to the C-1 to C-6' unit, and strongly suggest direct intact incorporation of the entire unit.

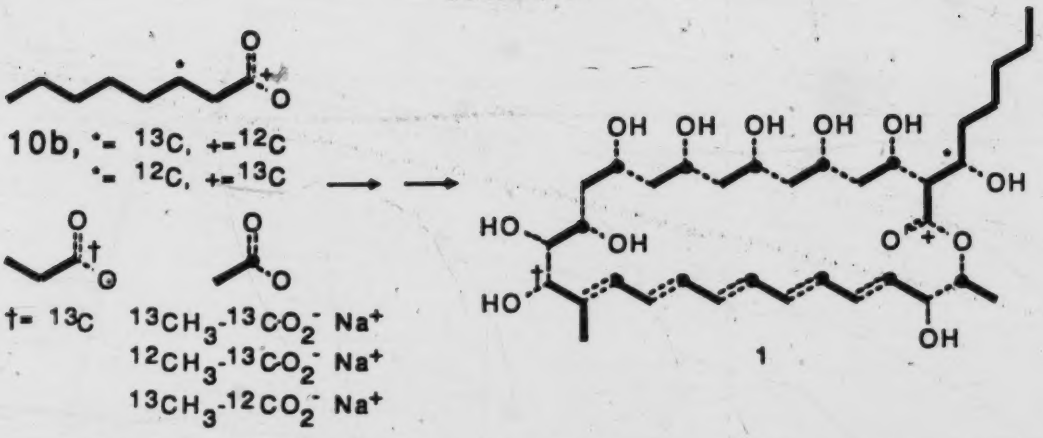
In all the feeding experiments described so far, incorporations are of the order of 2 to 5% (Table 1). However, incorporation of diethyl malonate is much higher than that of acetate (Figure 12g), despite the fact that malonate is converted to acetate prior to incorporation. These results, in general agreement with other biosynthetic experiments, may be interpreted in terms of poor transport of the labelled precursors to the enzymes that produce product. Transport may be inhibited by the cell wall, as seen by the use of broken cell extracts to effect incorporations not observed in whole cells; alternatively, transesterification of the labelled compound onto the enzyme may be inefficient.[227, 228]

Scheme 4.



The unusual specific incorporation of octanoate suggests the possibility of using chemically modified precursors to give modified antibiotics. The products might have enhanced or diminished biological activity, which could be correlated with the structural modification that has been "chemically engineered" into the antibiotic. However, such an experiment requires higher levels of incorporation than those obtained above so a more efficient precursor of the octanoate unit was sought.

Scheme 5.

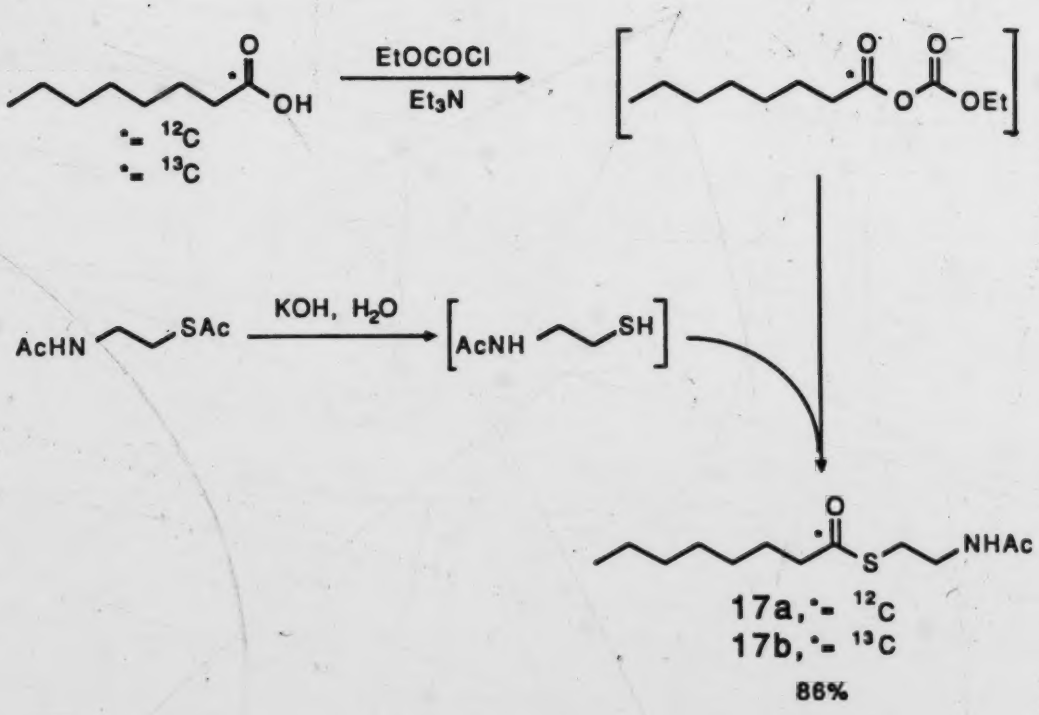


The success with diethyl malonate prompts investigation of incorporation of ethyl octanoate (**16a**). Ethyl [1-^{13}C]octanoate (**16b**) is readily prepared from [1-^{13}C]octanoic acid and ethanol with 1 equivalent of thionyl chloride. Although **16b** is incorporated specifically into **1**, the level of enrichment is low (Table 1).

S-Acyl-N-acetyl-cysteamines have recently been used with conspicuous success in incorporation studies with advanced putative intermediates in polyketide biosynthesis.[227, 229] These results prompt investigation of the incorporation of S-octanoyl-N-acetyl-cysteamine (**17a**), which may be prepared using the method of Kass and Brock.[230] Activation of octanoic acid with triethylamine and ethyl chloroformate generates the monoacyl carbonate, which reacts *in situ* with N-acetylcysteamine to give **17a** (Scheme 6). N-acetylcysteamine is itself generated *in situ* by selective hydrolysis of N,S-diacetylcysteamine. An analogous preparation from [1-^{13}C]octanoic acid gives the isotopomer **17b**. Again, however, **17b** incorporates into fungichromin (**1**) specifically, but only poorly (Table 1), possibly due to rapid *in vitro* hydrolysis to [1-^{13}C]octanoate in the medium.

Early literature descriptions of experiments designed to optimise the growth medium for fungichromin (**1**)[231] or filipin (**2**)[232] production describe much enhanced yields in the presence of fatty acids. Of the fatty acids studied, oleic acid appears to be the

Scheme 6.

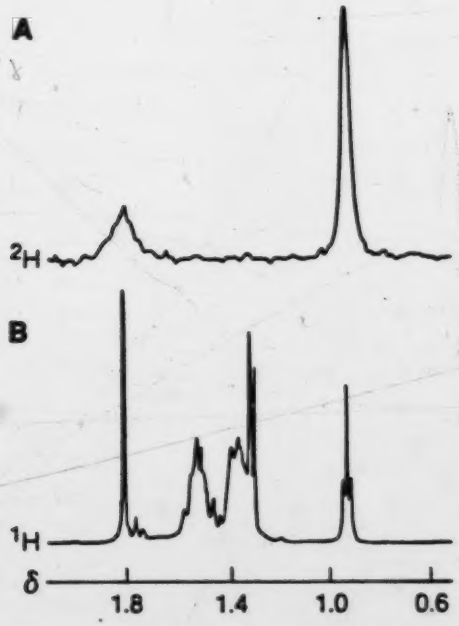


optimum. In the current work, Span 85 (a mixture whose principal component appears to be sorbitan tri-oleate) is used in the medium. Since [U-^{13}C]glucose is not incorporated into the octanoate unit of **1** (see above), Span 85 may be acting as the carbon source for this unit.

To test this hypothesis, a labelled precursor is required. Since Span 85 is not suitable for this purpose, it was hoped that a pure oleate derivative could replace the Span. Oleic acid or sodium oleate are not very suitable, due to agglomeration and detergent action. However, Dr. K. Arai has shown ethyl oleate to be as good as Span 85 as a fatty acid source for *S. cellulosae*. Dr. Arai has prepared ethyl [CD_3]oleate (**18a**).[233] Replacement of 10% of the ethyl oleate with the labelled compound gives fungichromin (**1**) containing 11 ± 2% of CD_3 groups at C-6', determined by ^{2}H NMR (Figure 13). Thus, oleate acts as the sole carbon source for C-6' of **1**, which may explain why oleate is required in order to

obtain good production of 1.[231]

Figure 13. (a) Partial ^{1}H NMR, and (b) Partial ^{2}H NMR spectra of fungichromin (1) enriched by incorporation of ethyl [CD_3]oleate.



These results strongly indicate that the C-1 to C-6' portion of fungichromin (1) is biosynthetically derived from C-11 to C-18 of oleate: this portion of oleate is cleaved to give octanoate, or a closely related 8-carbon derivative that is biosynthetically derived from octanoate. This species then incorporates intact into 1. Interestingly, the CD_3 group of ethyl [CD_3]oleate is also incorporated specifically at C-29 of 1 (Figure 13). Thus, oleate also appears to act as the source of ca. 50% of the propionate in *S. cellulosae*. The results are supported by the simultaneous incorporation of ethyl [U-^{14}C]oleate (18b). Oleate is known to be catabolised by β-oxidation to form dodec-3-enoate. After isomerisation to dodec-2-enoate, water undergoes a 1,4-addition to the unsaturated system to give 3-hydroxydodecanoate. Further β-oxidation, which stops at the C_8-stage in *S. cellulosae*, could account for formation of octanoate.

The detailed biosynthetic sources of all the carbon atoms of 1 are shown in Scheme 5.

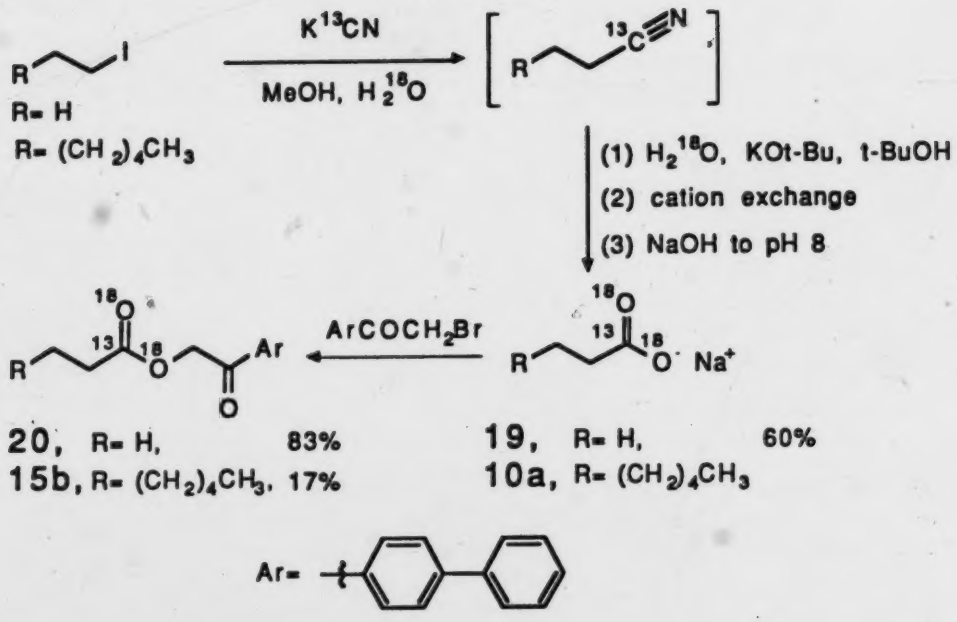
The next biosynthetic question relates to the origin of the oxygen atoms of 1. The Birch-Collie hypothesis suggests that oxygen atoms on carbons derived from C-1 of acetate will originate from acetate, since the carbon-oxygen bond remains intact through the condensation of acetate and malonate to give the β-ketoester, and in the subsequent reduction to the β-hydroxyester (Scheme 2).[196, 197] However, oxygen atoms at positions derived from C-2 of acetate must have a different biosynthetic origin.

Labelling experiments with both isotopes of oxygen, ^{17}O and ^{18}O, are documented in biosynthetic chemistry.[203] Oxygen-17 has a spin of 5/2, and can thus be directly detected by NMR; however, line widths are broad due to the quadrupole.[234] Oxygen-18 has no spin, but can be detected by an isotope shift in the attached α-, or in the β-carbon-13 signal.[235] These isotope shifts range from 10 to 55 ppb, depending on circumstances, but are larger for double bonds ($^{13}C=^{18}O$) than for single bonds, and are additive.[236] The isotope shift effect permits bond labelling. Thus, incorporation of [1-^{13}C, $^{18}O_2$]acetate into a natural product normally only gives a detectable isotope shift if at least one of the carbon-oxygen bonds remains intact. If both bonds are split, then scrambling of the isotopes with the unlabelled analogues normally occurs.

Sodium [1-^{13}C, $^{18}O_2$]octanoate (**10a**) was readily prepared (Dr. H. Noguchi) by reaction of heptyl iodide with potassium [^{13}C]cyanide (Scheme 7) to give [1-^{13}C]octanonitrile, using the method developed for the propionate analogue by Cane *et al.*[237, 238] Hydrolysis with potassium *t*-butoxide in $H_2{}^{18}O$ gives [1-^{13}C, $^{18}O_2$]octanoic acid, isolated as the sodium salt **10a**.

Derivatisation of a small sample of **10a** as the *p*-phenylphenacyl ester (**15b**), using the method of Cane *et al*,[237] allows determination of the isotopic purity (Scheme 7). Analysis by mass spectrometry gives molecular ions, the relative intensities of which give labellings of 100% ^{13}C, and 92% ^{18}O/site.

Scheme 7.



Incorporation of **10a** into fungichromin (**1**) gives a sample whose partial ^{13}C NMR spectrum is shown in Figure 14. Upon expansion of the region for C-1 of **1**, an ^{18}O isotopically-shifted resonance is observed. The isotope shift is ca. 40 ppb (Table 2), and indicates that the carbonyl oxygen is labelled. This result thus shows that one of the carbon-oxygen bonds of octanoate remains intact during incorporation into **1**, and becomes the carbonyl oxygen-carbon bond at C-1. An analogous experiment with sodium [1-^{13}C, $^{18}O_2$]acetate gives isotope shifts at all the expected positions derived from C-1 of acetate (Table 2). In order to test whether the C-OH bond at C-15 of **1** remains intact from propionate, sodium [1-^{13}C, $^{18}O_2$]propionate (**19**) may be fed. The synthesis of Cane *et al* [237, 238] was used. Substitution of ethyl iodide with potassium [^{13}C]cyanide gives [1-^{13}C]propionitrile, which upon hydrolysis with potassium *t*-butoxide in $H_2{}^{18}O$ gives [1-^{13}C, $^{18}O_2$]propionic acid, isolated as the sodium salt **19** (Scheme 7). Derivatisation as the *p*-phenylphenacyl ester **20**[237] shows the isotopic labelling to be 96%^{13}C, 86% ^{18}O/site. Incorporation of **19** into **1** gives an isotope shift only at C-15 (Table 2).

Figure 14. C-1 signal of fungichromin (1) labelled with sodium [1-^{13}C, $^{18}O_2$]octanoate (**10a**).

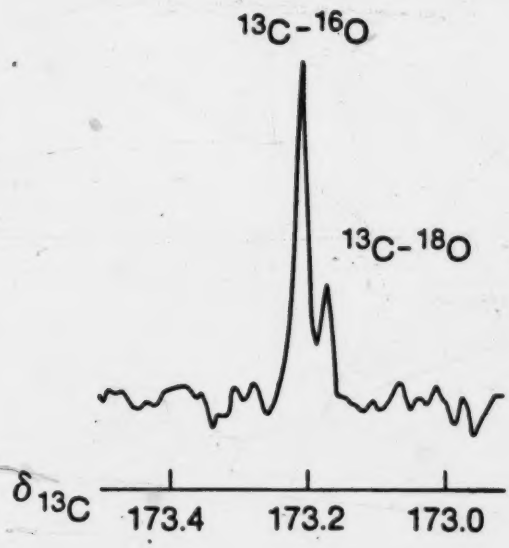


Table 2

^{18}O isotopically shifted resonances in fungichromin (1) from labelled precursors

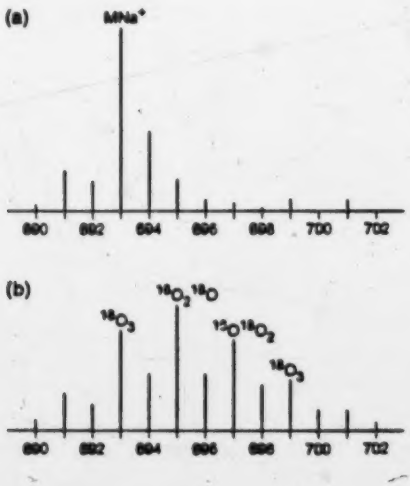
Carbon	^{13}C δ (ppm)	^{18}O isotope shift (ppb)	Precursor
1	172.98	40	a
15	80.43	20	b
14	78.31	16	c
27	75.25	28	d
9	74.20	20	d
5	74.08	20	d
7	73.92	20	d
3	73.41	20	d
26	73.25	10	c
1'	72.59	12	c
11	71.45	20	d
13	70.34	20	d

[a]Sodium [1-^{13}C, $^{18}O_2$]octanoate (**10a**). [b]Sodium [1-^{13}C, $^{18}O_2$]propionate (**19**).

c $^{18}O_2$ gas. [d]Sodium [1-^{13}C, $^{18}O_2$]acetate.

Three oxygen atoms remain biosynthetically unaccounted for. These atoms are at positions derived from C-2 of acetate. Incubation of *S. cellulosae* in a closed atmosphere of $^{18}O_2$ gas (ca. 50% ^{18}O) gives 1 with small isotope shifts at all three positions (Table 2). However, the large natural broadness of the ^{13}C NMR signals at those sites, the diminished sensitivity in this experiment[203] due to the lack of enrichment of the site by ^{13}C, and the low yield of 1 make positive identification of these isotope shifts difficult. Further confirmation was obtained from the fast-atom bombardment mass spectrum (FAB-MS) of the resultant 1 (Figure 15). Although the signal-to-noise is not good, the data suggest incorporation of 0, 1, 2 or 3, but not more, ^{18}O atoms per molecule, and a best fit is obtained for 35% ^{18}O/site.

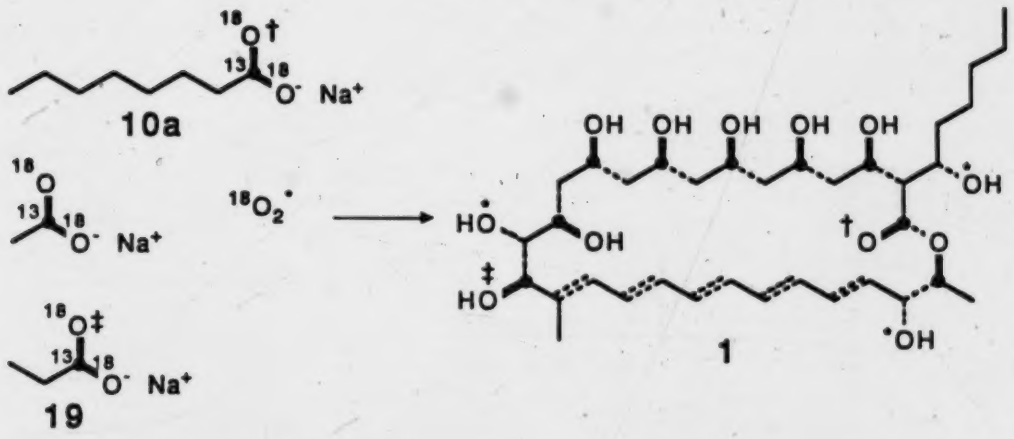
Figure 15. Fast-atom bombardment mass spectrum of fungichromin (1) for the [M + Na]$^+$ region: (a) natural abundance, (b) enriched by $^{18}O_2$ gas.



The biosynthetic results of oxygen-18 incorporation experiments are summarised in Scheme 8. The results are consistent with a polyketide biosynthesis from 12 acetate units, 1 propionate unit and an octanoate unit. The remaining 3 oxygen atoms are probably

introduced by aerobic oxidation at some point during the late stages of biosynthetic transformation.

Scheme 8.



Next elucidation of the biosynthetic origin of the hydrogen atoms of 1 was attempted. Such elucidation is often accomplished by incorporation of sodium [2-^{13}C, D_3]acetate into natural products, followed by location of deuterium from its isotope shift in the ^{13}C NMR spectrum.[203] However, such an incorporation experiment with 1 leads to material that contains no significant amount of deuterium. Since deuterium must be incorporated via 2-[$^2H_2$]malonate in every acetate-derived unit but the starter,[203] deuterium may be lost by exchange with the aqueous medium. To test this hypothesis, *S. cellulosae* was grown in medium containing D_2O. The resultant fungichromin (1) has extensive deuterium incorporation, as shown by deuterium NMR. However, deuterium was absent at positions 6', 5', 4', 3' and (at least partially) 2', as shown by the absence of multiple isotope shifts at these positions in the ^{13}C NMR spectrum. This result is expected, since these positions derive from oleate via octanoate.

The loss of deuterium from the starter acetate unit in the experiment with sodium [2-^{13}C, D_3]acetate is less common, because such deuterium loss normally requires exchange

in acetate rather than in malonate.[203] Intact incorporation within one acetate unit of three deuteriums is often used as evidence for a polyketide starter unit, since all other units must come from malonate and can thus only incorporate a maximum of 2 deuterium atoms. In order to show that C-28 and C-27 in 1 constitute a starter unit for the polyketide chain, diethyl [2-^{13}C]malonate was incorporated in the hope that it would label the units that come from malonate more than the starter unit, which comes from acetate. However, C-28 in 1 is labelled as efficiently as are all the other sites in this experiment. Dr. K. Arai has shown that *S. cellulosae* grown in media containing [2-^{13}C, D_3]acetate in the absence of added fatty acid produces fatty acids with no deuterium. Clearly, *S. cellulosae* can rapidly interconvert acetate and malonate, and thus exchange deuterium out of [2-^{13}C, D_3]acetate.

These experiments provide more conclusive evidence for the polyketide biogenesis of polyene antibiotics than was possible in earlier studies using radioisotopes, since the site of labelling is readily determined. Fungichromin (1) derives from head-to-tail condensation of twelve acetate units, one propionate unit and an intact octanoate unit. The incorporation of octanoate was only the second example of intact incorporation of a long-chain fatty acid with greater than 4 carbon atoms as a unit in a polyketide-derived metabolite. The octanoate derives from degradation of oleate, rather than from biosynthesis from acetate.

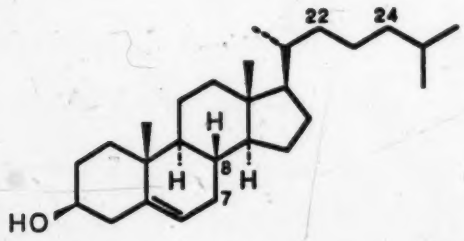
The techniques developed for assignment of the ^{13}C NMR signals of 1 should prove useful in assignment and structural elucidation of other polyene antibiotics; the novel method for determining interunit connectivity may prove useful in assignment of polyketides in general.

Finally, the development of a purification method for polyenes using reverse-phase medium-pressure liquid chromatography may prove useful as a complementary technique to the much-used countercurrent distribution.

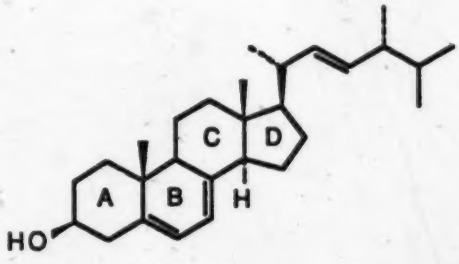
Synthesis of Sterols for Structure-Activity Correlations

One approach to understanding the role of sterols in the mechanism of action of the polyene antibiotics involves determining structure-activity relationships for the sterol. While a large number of such investigations are reported in the literature (see introduction), many involve the use of readily available sterols, for which there are no systematic trends in the structural modifications, making the detailed analysis of the role of each structural feature difficult.

Cholesterol (3) and ergosterol (4) differ in three structural features: ergosterol possesses unsaturation in the B-ring between carbons 7 and 8, and in the side-chain, between carbons 22 and 23; and ergosterol has a methyl group at carbon 24. The observed selectivity for binding of ergosterol in favor of cholesterol is apparently due to one or more of these molecular features. The other six sterols (Figure 16) which differ in the presence or absence of each functionality, were therefore chosen for synthesis. The interaction of each sterol with polyenes could then be investigated.



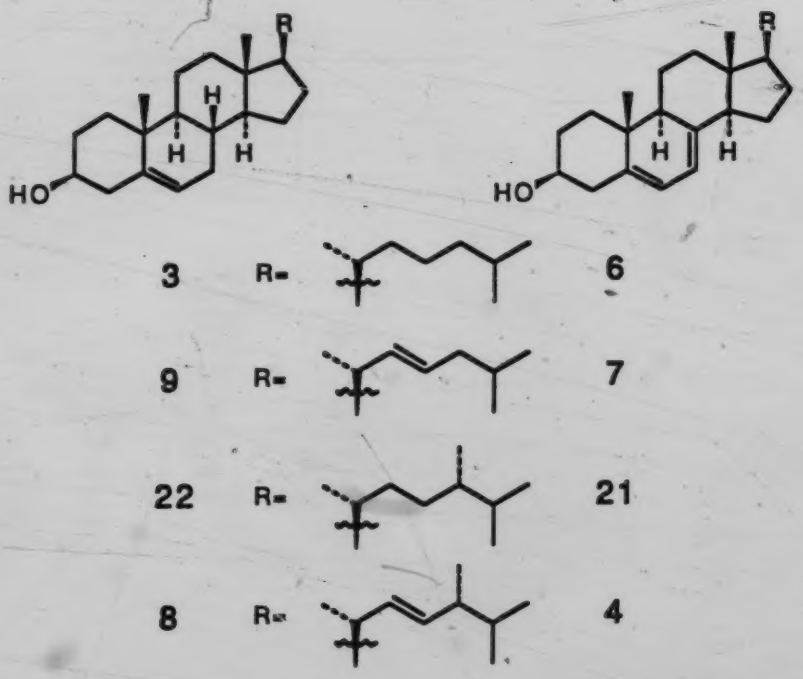
3



4

The first synthetic goal was cholesta-5,7-dien-3β-ol (6). This compound has aroused particular interest since it is the naturally occuring pro-vitamin for vitamin D_3.[239] Synthetically, 6 has been prepared by allylic oxidation of cholesterol derivatives, to give substituted 7-hydroxy-cholesterols, which are subsequently eliminated to afford a mixture

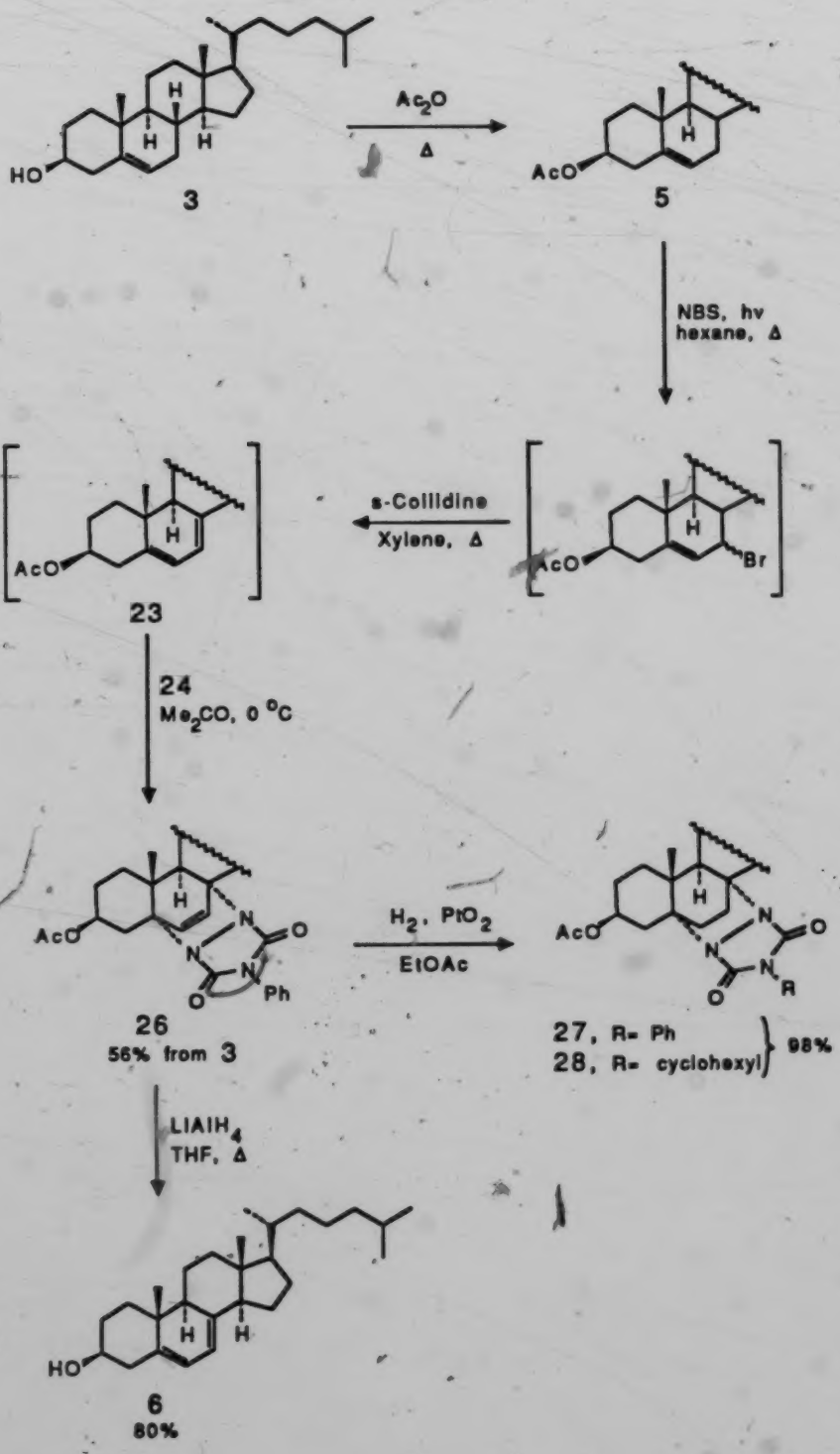
Figure 16. Synthetic target sterols.



of 4,6- and 5,7-dienes.[240] Alternatively, allylic bromination by free-radical halogenating agents, which occurs predominantly at C-7, followed by dehydrohalogenation, also gives a similar mixture of dienes.[241] Despite the existence of a considerable literature on the latter more efficient process, in which variations in hydroxyl protecting group,[241, 242] halogenating agent,[243, 244] reaction solvents,[241, 242] base,[241, 242, 245, 246] and recrystallisation solvent,[247] have been thoroughly investigated, the optimum reported yield is only 43%,[248] while most yields are of the order of 20%. While such a synthesis of **6** is acceptable from cholesterol, a higher-yielding process was sought, since the ability to effect such a transformation would prove useful in synthesis of the other target compounds.

Cholesterol (**3**) reacts readily with acetic anhydride and pyridine[249] to afford acetate **5**,[250] which is now suitably protected for further transformation (Scheme 9). Photolysis of **5** and N-bromosuccinimide in hexane gives the unstable allylic bromides,

Scheme 9.

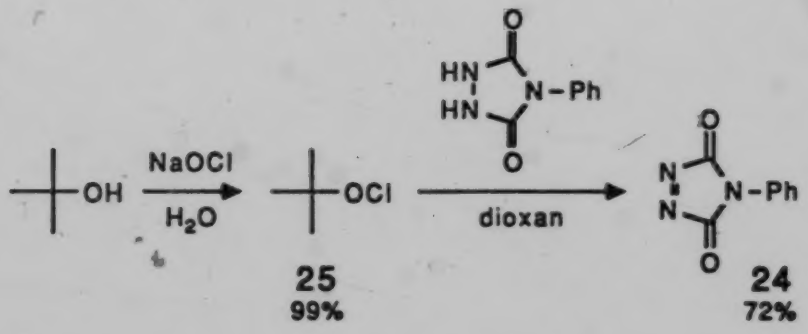

HO
3
Ac_2O
Δ
AcO
5
NBS, hv
hexane, Δ
Br
s-Collidine
Xylene, Δ
23
24
Me_2CO, 0 °C
Ph
26
56% from 3
H_2, PtO_2
EtOAc
R
27, R= Ph
28, R= cyclohexyl
98%
$LiAlH_4$
THF, Δ
6
80%

which eliminate in the presence of *s* -collidine (2, 4, 6-trimethylpyridine) to give a mixture containing the desired 3β-acetoxycholesta-5,7-diene (23) and several other sterols.[241] NMR shows that the mixture contains up to approximately 60% of **23**. However, all attempts at recrystallisation following literature[241, 242, 244] or other procedures fail to give **23** in satisfactory yield or of sufficient purity. Furthermore, the compounds are not readily separable by column or argentation chromatography. Therefore, a chemical separation of this material was attempted.

One possible approach to separation involves the use of the Diels-Alder reaction to selectively convert the 5,7-diene system to a separable derivative. The 5,7-diene possesses the required cisoid geometry for the Diels-Alder reaction while the side-product, 3β-acetoxycholesta-4,6-diene,[241] is locked into the unreactive transoid geometry. Separation of unreacted starting material would also be facile, since no reaction is expected.

One of the most common protecting groups in steroid chemistry for the B-ring diene system is the N-phenyltriazoline-3,5-dione derivative.[251] The precursor, N-phenyltriazoline-3,5-dione (**24**),[251] is readily prepared from N-phenylurazole by oxidation with *t* -butyl hypochlorite (**25**)[252] (Scheme 10). Compound **24** reacts rapidly and quantitatively with steroidal dienes at or below room temperature to afford Diels-Alder adducts.[253]

Scheme 10.



25
99%

24
72%

The partially purified mixture of acetates from the dehydrobromination reaction condenses with **24** to give the readily separable derivative **26**[254] (Scheme 9). The reaction is readily followed, since **24** is bright red, while the derivative is colorless: since the reaction is instantaneous at 0 °C, titration to determine the amount of 5,7-diene is possible. The titration results agree very well with the isolated yields of **26**. The optimum photolysis time is therefore easily determined without product isolation, as could be the optima for other reaction conditions.

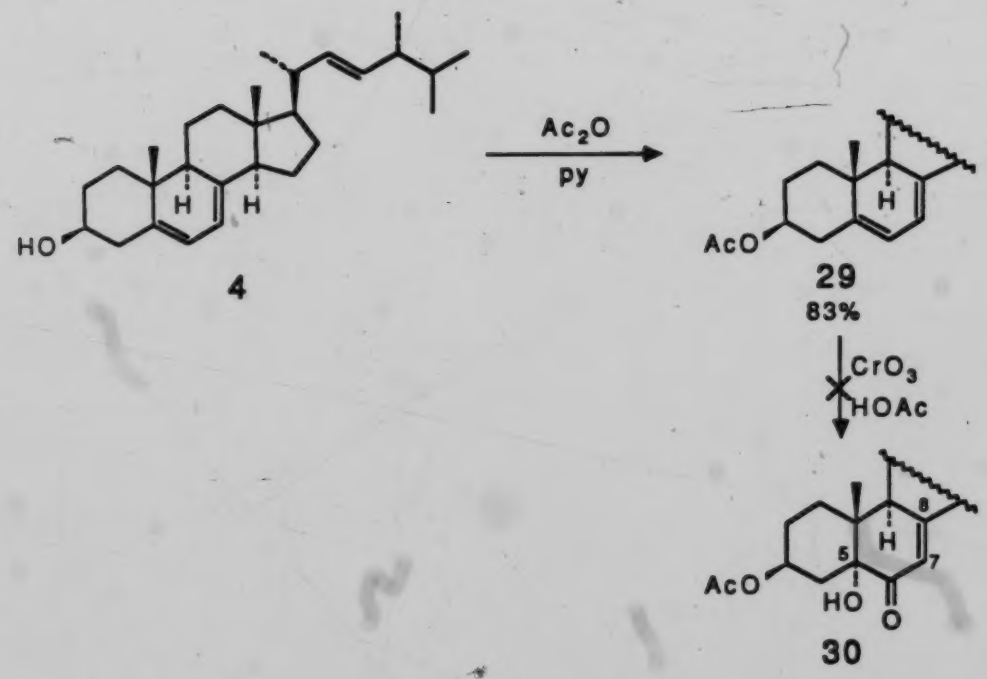
The N-phenyltriazoline-3,5-dione derivative represents a useful protected form of the diene for further synthetic transformations.[253, 254] Alternatively, it may be readily converted back to the diene under a variety of conditions.[253, 255] Thus, reaction of **26** with lithium aluminium hydride in THF at reflux[253] readily removes both the hydroxyl and diene protecting groups to afford the desired **6** in 45% overall yield from **3** (Scheme 9).

Adduct **26** and other triazolinedione derivatives (see later) fail to give satisfactory analyses for nitrogen, perhaps due to facile conversion to nitrogen gas following a retro-Diels-Alder reaction upon heating. Furthermore, although a weak molecular ion is observed in the ammonia chemical ionization mass spectrum, attempts to obtain an exact mass under electron impact conditions were unsuccessful: a strong peak due to retro-Diels-Alder fragmentation is observed. The 6,7-dihydro derivative **27** was expected to be less prone to fragmentation in the mass spectrometer. Compound **26** reacts readily with hydrogen and Adam's catalyst (platinum dioxide)[256] to give **27** (Scheme 9). The N-cyclohexyl analogue **28** is a by-product that is not readily separable. However, high-resolution electron impact mass spectrometry gives satisfactory molecular ions, thus confirming the molecular formulas of **27** and **28**, and hence of **26**.

The second synthetic goal is (22*E*)-ergosta-5,22-dien-3β-ol (**8**), a common naturally occuring phytosterol (brassicasterol)[257] present in rape seed.[258, 259] The related oxidised sterols, brassinolides, are important plant growth stimulators.[260-262] Several approaches to synthesis of **8** have been reported which involve reduction of the diene

system of ergosterol. Burawoy[263] found that oxidation of ergosteryl acetate (29) with chromium trioxide in acetic acid afforded a material to which structure 30 was assigned (Scheme 11). Barton and Robinson[264] observed that reaction of 30 with metal-ammonia solutions caused selective reduction of the 7-8 double bond; a Wolff-Kishner type reduction of the resulting sterol regenerated the 5-6 double bond to afford a derivative of 8.

Scheme 11.



Ergosterol (4) reacts with acetic anhydride and pyridine to afford acetate 29. Oxidation under the conditions described by Barton and Robinson[264] gives a material that agrees well in melting point and optical rotation with the reported values. The infrared, ultraviolet, mass and high-field proton NMR spectra further support the proposed structure. However, carbon-13 NMR reveals a mixture of isomers in a ratio of ca. 1 : 1. The uncertainty in stereochemistry implied by this result, and the observation that reduction of this material with lithium in ammonia leads to a complex mixture of products by TLC,

which are not readily separable, suggests an alternative approach.

More recently, Anastasia *et al* [265] reported partially selective reduction of the N-phenyltriazoline-3,5-dione derivative of ergosteryl acetate (**31**) to give a mixture of the desired (22*E*)-ergosta-5,22-dien-3β-ol (**8**) and the corresponding (22*E*)-5α- and 5β-7,22-dienes. It appeared that **8** might be accessible through this approach.

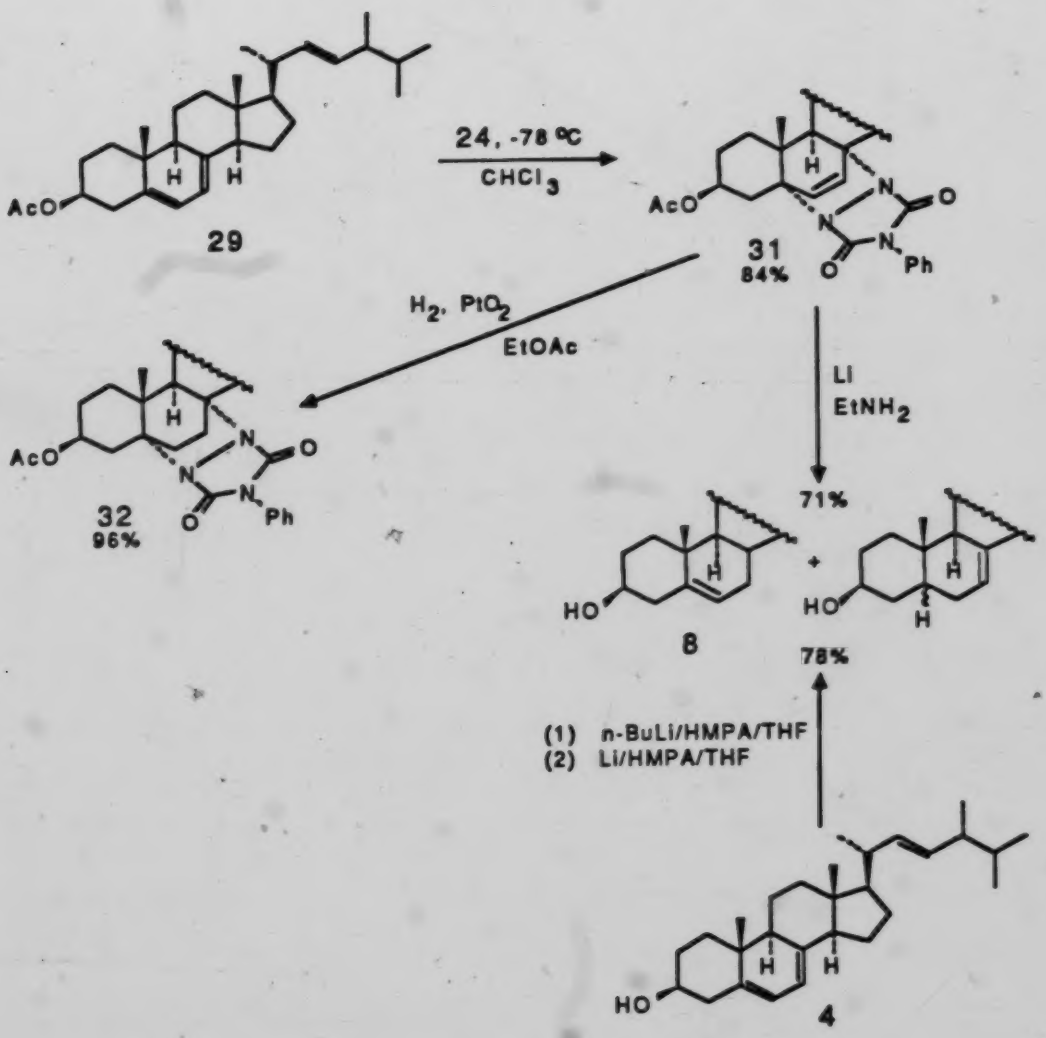
Acetate **29** reacts readily with N-phenyltriazoline-3,5-dione (**24**) in acetone at -78 °C to afford adduct **31** (Scheme 12).[253] Catalytic reduction of **31**, analogous to that of **26** described above, gives the tetrahydro derivative **32**, which again affords a satisfactory exact mass for the molecular ion. Investigation of several catalytic systems reveals that reduction of the 6-7 double bond is competitive with reduction of that in the side-chain, as has been reported.[256] Unfortunately, this precludes use of **31** for synthesis of ergosta-5,7-dien-3β-ol (**21**) by hydrogenation followed by deprotection of the B-ring diene.

Adduct **31** reacts with lithium in ethylamine at reflux to afford an approximately equimolar mixture of (22*E*)-ergosta-5,22-dien-3β-ol (**8**) and (22*E*)-5α-ergosta-7,22-dien-3β-ol, containing smaller amounts of (22*E*)-5β-ergosta-7,22-dien-3β-ol and other sterols.

Recently, Barton *et al* have reported direct reduction of ergosterol (**4**) to a mixture of the (22*E*)-5,22- and (22*E*)-7,22-dienes.[266] Of the several variations reported, generation of ergosterol anion with n-butyllithium followed by reduction with lithium in HMPA , THF, and diisopropylamine, was most efficient. This route (Scheme 12) leads more directly to the desired product, and proves to be more desirable in this case.

The mixture of isomeric sterols is not readily chromatographically separable. A chemical separation using the method of Barton *et al* [266] is therefore attractive; also, some of the intermediates involved may be converted to other target sterols, as described below.

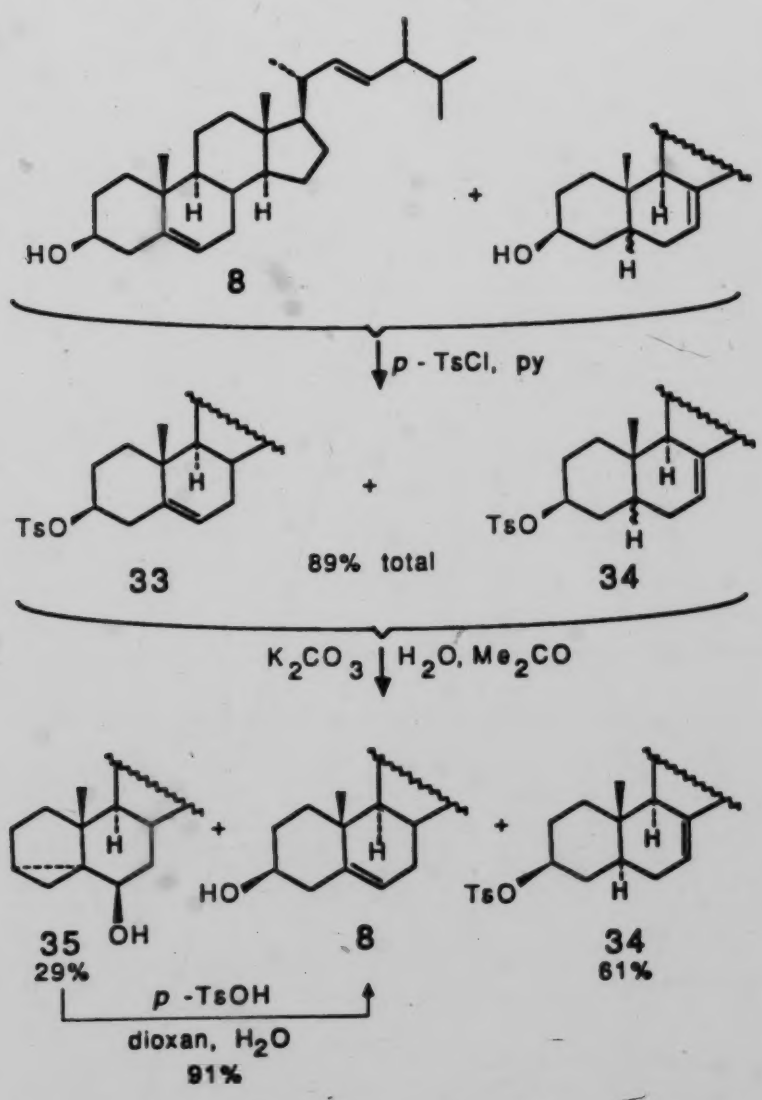
Scheme 12.



Reaction of the mixture of sterols with *p*-toluenesulfonyl chloride in pyridine affords a mixture of sterol *p*-toluenesulfonates **33** and **34** (Scheme 13). Hydrolysis under kinetically controlled conditions causes the desired (22*E*)-5,22-diene sulfonate **33** to form i-sterol **35**, while the (22*E*)-7,22-diene sulfonates fail to react. Small amounts of the normal sterol **8**, the thermodynamic product of solvolysis, also form. The i-sterol **35** is readily separable from the reaction mixture. Pure 5α-toluenesulfonate **34** is also readily

obtained by recrystallisation of the unreacted material. Following separation, **35** readily converts to the desired product, (22*E*)-ergosta-5,22-dien-3β-ol (**8**), with *p*-toluenesulfonic acid in warm aqueous dioxane.[267]

Scheme 13.



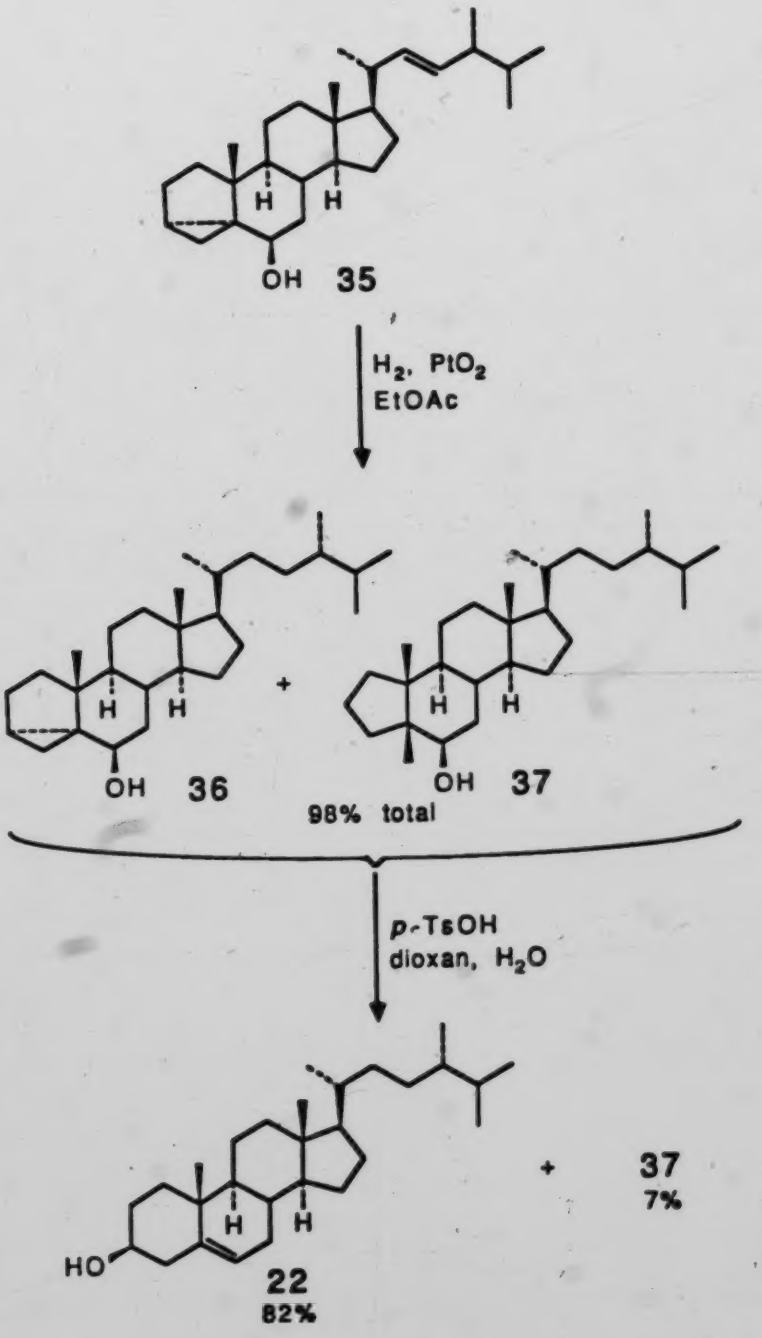
The third target sterol is ergost-5-en-3β-ol (**22**).[267] Compound **22** is a naturally occurring sterol found in marine sponges[268, 269] and in corals.[270] The availability of i-sterol **35** from the synthesis above suggests catalytic hydrogenation, followed by i-sterol to

sterol conversion, as a facile route to this compound. Indeed, a modification of such a route has been described.[267] Other synthetic routes reported in the literature include direct catalytic hydrogenation of dienol **8**,[258] and an elegant preparation from fucosterol.[271]

Catalytic hydrogenation of **35** over platinum dioxide rapidly reduces the side-chain double bond to afford **36** (Scheme 14). The product contains small amounts of an impurity, which varies somewhat in amount, but is typically 5 to 10% of the total mass. This by-product always forms when hydrogenation of the double bond is taken to completion, a synthetic requirement since **35** and **36** are not readily separable. The impurity is not readily separated from the desired product. However, when the i-sterol to sterol rearrangement is effected, the impurity fails to react and is readily removed. Spectral investigation reveals it to be 6β-hydroxy-5β-methyl-4-nor-ergostane (**37**), which probably forms by catalytic hydrogenation of the cyclopropane ring in **35** and/or **36**. Such reductions of cyclopropanes[272] are well known to occur at the least hindered bond. The i-sterol **36** readily undergoes acid-catalysed rearrangement to the desired thermodynamic product **22** (Scheme 14).

The fourth synthetic goal is ergosta-5,7-dien-3β-ol (**21**), which is of interest as a potential precursor to ergosterol and to vitamin D, and which also occurs in many fungi[273] and a mutant yeast.[274] Several routes have been reported for synthesis of **21**. These routes involve protection of the B-ring diene by Diels-Alder reaction, followed by catalytic reduction of the side-chain double bond. Curry *et al*[275] describe a route using the cyclic peroxide of ergosteryl acetate, derived from addition of oxygen to the diene. The moderate yields are very sensitive to the exacting conditions of several of the steps. Kircher and Rosenstein[276] describe an improvement on early inefficient routes[277, 278] from the maleic anhydride adduct. However, ene reactions compete during formation of the adduct, and catalytic hydrogenation may proceed beyond the desired extent. Furthermore, the elevated temperatures required to effect the retro-Diels-Alder reaction cause side-products to form.

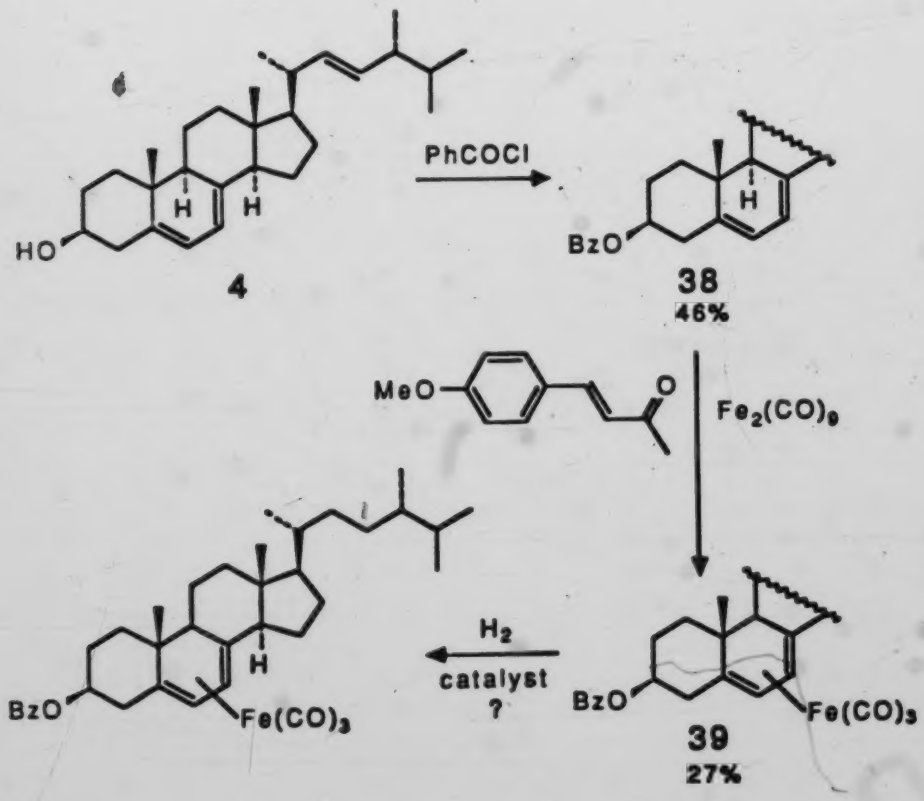
Scheme 14.


OH 35
H2, PtO2
EtOAc
OH 36
+
OH 37
98% total
p-TsOH
dioxan, H2O
HO
22
82%
+ 37
7%

Barton *et al* [256] have published several routes to **21**. The authors suggest that the most attractive route involves catalytic hydrogenation of the iron tricarbonyl adduct, since N-phenyltriazoline-3,5-dione adducts are not well suited to this purpose. This route appears attractive, since it involves only four synthetic steps.

Ergosterol reacts with benzoyl chloride to afford the benzoate **38**. Ester **38** undergoes an exceedingly slow condensation (27% conversion in 1 month) with di-iron nonacarbonyl catalysed by *p* -methoxybenzylideneacetone to afford the adduct **39** (Scheme 15). Extensive purification allows isolation of pure product, which gives satisfactory spectral data; however, the data does not agree exactly with that of Barton *et al* :[256] infrared bands at ca. 1600 cm^{-1} reported by Barton are clearly absent in the compound prepared in these laboratories. The source of this discrepancy is at present unclear.

Scheme 15.



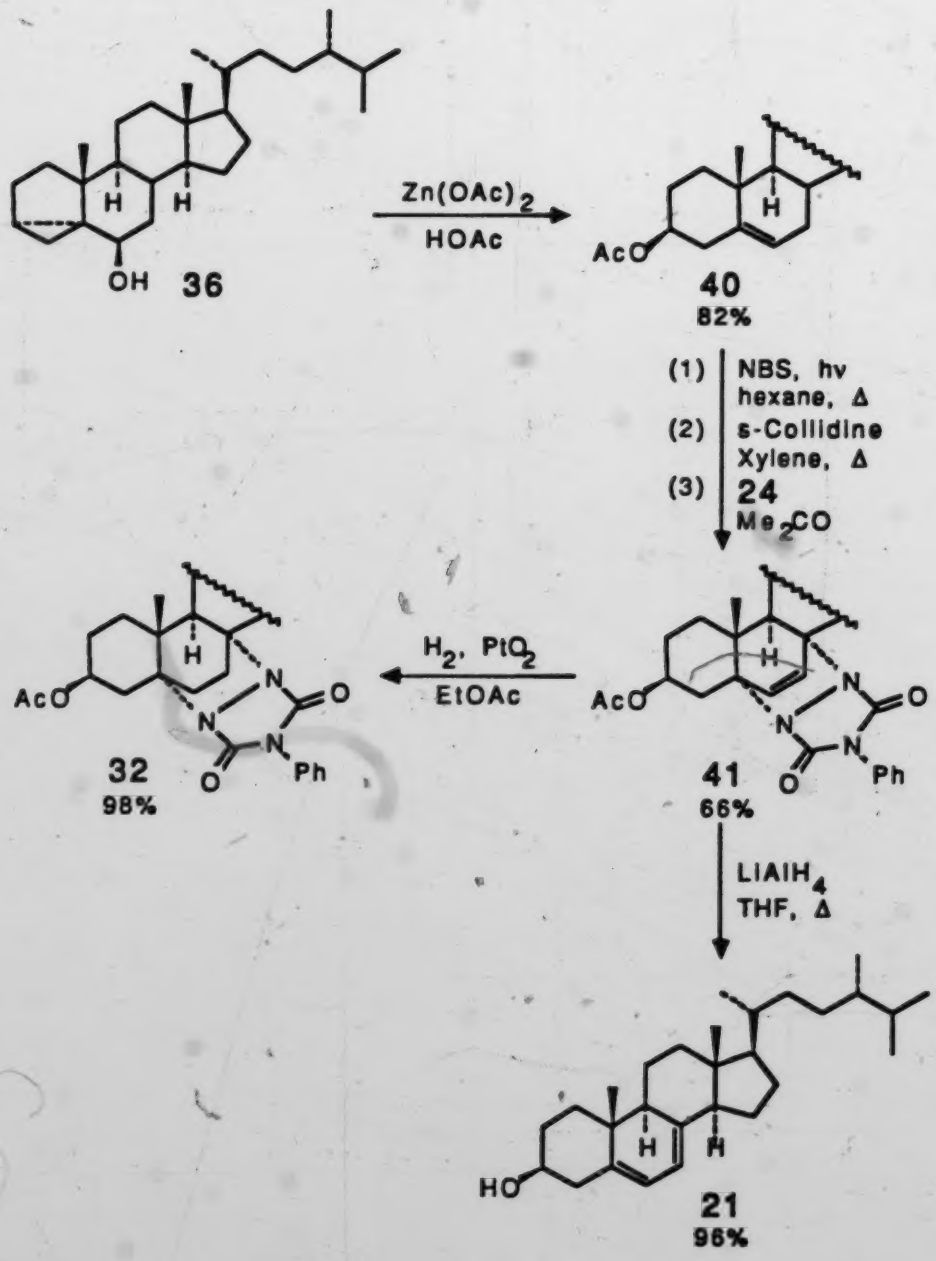
Catalytic hydrogenation of **39** over platinum dioxide with benzyldimethylsilane as catalyst for 1 month, using the method of Barton *et al*,[256] gives a complex mixture of products. Mass spectrometry and NMR indicate that no more than ca. 33% of the desired product is present. Kircher and Rosenstein[276] also report failure to effect this transformation. This route was thus discarded in favour of more promising approaches.

While the latter two reactions were in progress, the development of successful methodology for introduction of the B-ring diene functionality into cholesterol described above prompted investigation of a similar route to ergosta-5,7-dien-3β-ol (**21**). Reaction of i-sterol **36** with zinc acetate in acetic acid following the procedure of Thompson *et al*[267] readily furnishes 3β-acetoxyergost-5-ene (**40**) (Scheme 16). The bromination-dehydrobromination and triazolinedione adduct formation sequence described for 3β-acetoxycholest-5-ene (**5**) then allows efficient (66% yield) formation of adduct **41**. Catalytic hydrogenation of **41** affords derivative **32**, identical in all respects to material prepared from **31**. Conversion of **41** to the desired ergosta-5,7-dien-3β-ol (**21**) proceeds in 96% yield upon reduction with lithium aluminium hydride. The overall procedure thus represents another example of the efficient conversion of sterols possessing a 5-6 double bond into the corresponding 5,7-dienes by this novel technique.

The syntheses of the first four sterols require only suitable manipulation of functional groups to provide the product. However, for the remaining targets, (22*E*)-cholesta-5,22-dien-3β-ol (**9**), a naturally occuring sterol in marine algae[279] and the Alaskan king crab,[280] and (22*E*)-cholesta-5,7,22-trien-3β-ol (**7**), no precursor that can be converted to product in such a fashion is readily available. Thus, literature approaches to synthesis of **9**,[281-286] **7**,[287, 288] and other related sterols involve removal of the steroidal side-chain followed by Wittig reaction[282-292] to generate the side-chain double bond. One such approach involves use of the protected aldehyde **42** (Scheme 17).[282, 286, 288-293]

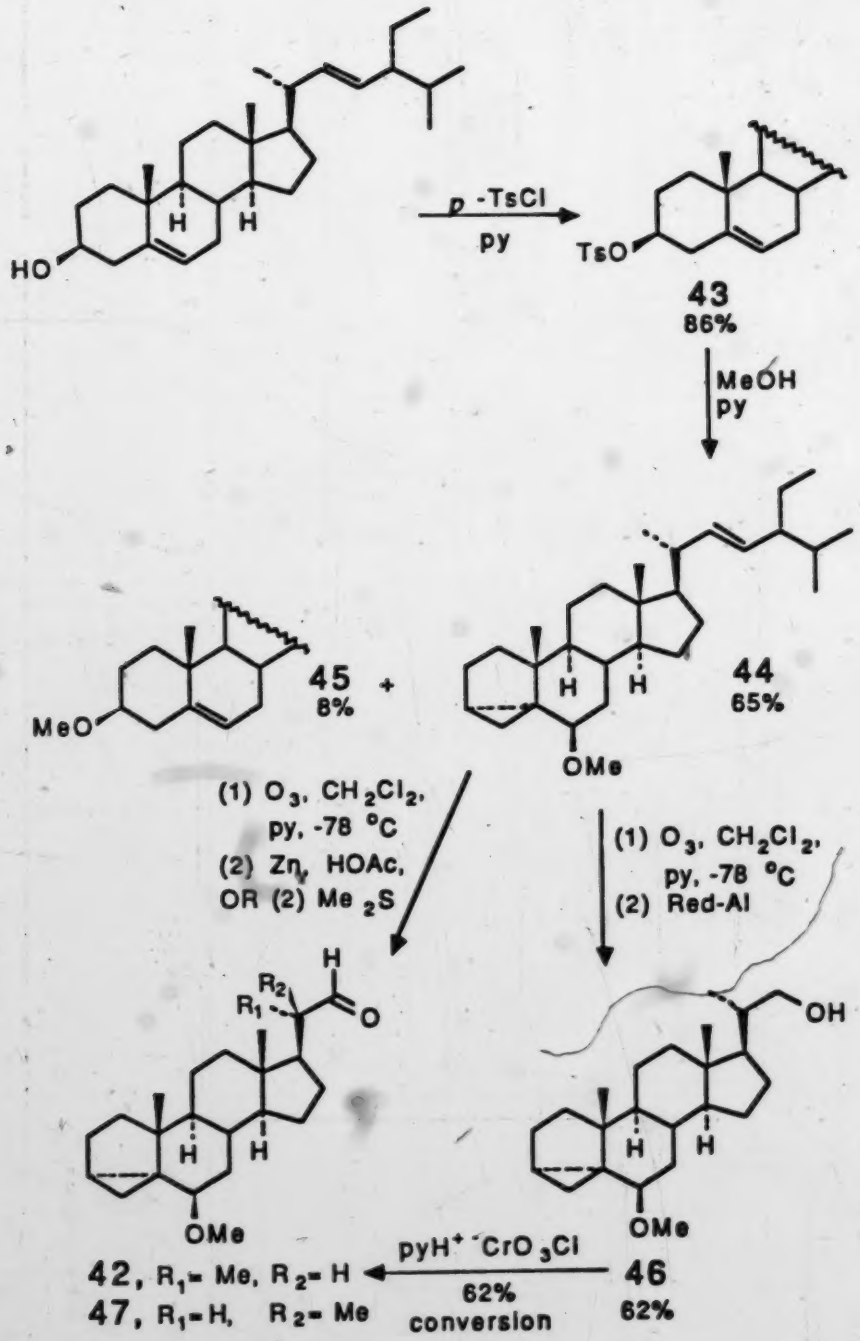
Using the procedure of Partridge *et al*[293] (Scheme 17), reaction of stigmasterol

Scheme 16.



with *p*-toluenesulfonyl chloride in pyridine affords toluenesulfonate **43**. Solvolysis of **43** under kinetic conditions affords i-methyl ether **44**, along with some of the readily separable thermodynamic product, **45**. The nuclear double bond is now suitably protected

Scheme 17.


HO
H
H
p -TsCl
py
TsO
43
86%
MeOH
py
MeO
45
8%
+
44
65%
OMe
(1) O3, CH2Cl2,
py, -78 °C
(2) Zn, HOAc,
OR (2) Me2S
(1) O3, CH2Cl2,
py, -78 °C
(2) Red-Al
R2
R1
O
OH
pyH+ CrO3Cl
62%
conversion
42, R1= Me, R2= H
47, R1=H, R2= Me
46
62%

for cleavage of the side-chain by ozonolysis. Thus, ozonolysis of **44** in dichloromethane and pyridine at -78 °C, followed by reductive work-up with sodium *bis*-(2-methoxy-ethoxy)aluminium hydride (Red-Al), gives alcohol **46** in 62% yield. It proves necessary to perform the ozonolysis in several separate vessels when working on a large scale. When one large scale ozonolysis is performed, yields are considerably lower, perhaps due to slow attack by ozone on the saturated C-H bonds of the sterol during the prolonged reaction. Work-up of the ozonolysis reaction with zinc and acetic acid following the procedure of Hutchins *et al*,[282] or with dimethyl sulfide, results in aldehyde **42**. However, the product is contaminated with varying amounts of aldehyde **47**,[284] probably derived from epimerisation at C-20 during work-up. The mixture of aldehydes is not readily separable. Oxidation of alcohol **46** with pyridinium chlorochromate under the conditions described by Corey and Suggs[294] affords the aldehyde in high yield, allowing for recovery of unreacted starting material, and is therefore the more attractive route.

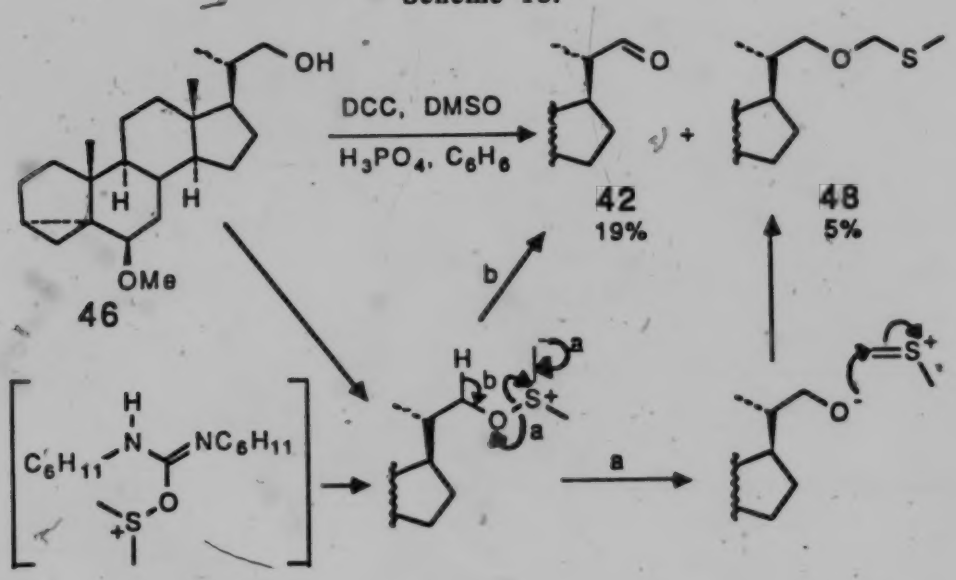
In all cases, close examination of the proton or carbon NMR of aldehyde **42** prepared by oxidation reveals contamination by epimer **47**,[284] which is always present at a level of 2 to 3%. Since the starting stigmasterol is only ca. 90% pure, the epimeric aldehyde **47** may arise in this case from contamination by the 22-epimer of stigmasterol, which is known to occur naturally. The other major impurity in commercial stigmasterol is the 22-dihydro analogue which is removed in the synthesis after ozonolysis, since it fails to react.[293]

Oxidation of **46** for prolonged times gives rise to unidentified impurities that are not readily separated from aldehyde **42**. Thus, the optimum conditions for this step involve incomplete conversion, followed by isolation and repeated oxidation. An alternative, higher-yielding oxidation method was therefore sought. One such mild route is the Moffatt oxidation.

Alcohol **46** reacts readily under typical Moffatt conditions, in benzene, with dicyclohexylcarbodiimide, dimethyl sulfoxide, and phosphoric acid, to give aldehyde **42**

(Scheme 18). However, examination of the reaction mixture by TLC reveals a novel compound, with an R_f close to that of the aldehyde, which forms at approximately the same rate. Isolation and spectral analysis shows this material to be sterol analogue **48**. Compound **48** presumably arises as shown in Scheme 18, a side-reaction which is well precedented in Moffatt oxidations.[295, 296] Since separation of **48** from **42** appears to be potentially problematic on a large scale, oxidation with pyridinium dichromate seems to be the most attractive route to aldehyde **42**.

Scheme 18.

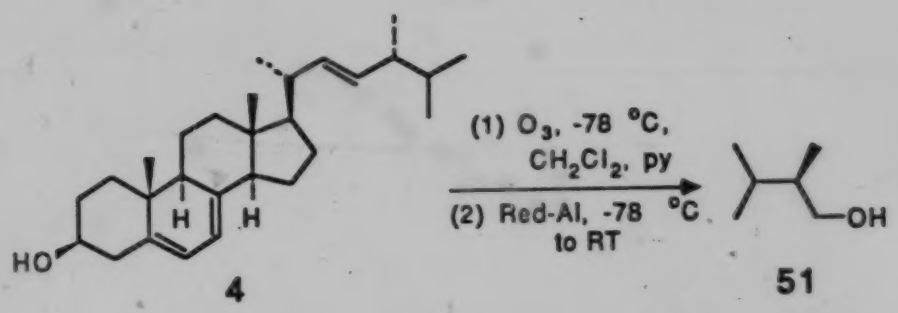


With the steroidal aldehyde available for Wittig coupling, phosphonium salts must be prepared. Two such compounds, (3-methylbutyl)triphenylphosphonium iodide (**49**)[285] and ((2*R*)-2,3-dimethylbutyl)triphenylphosphonium iodide (**50**),[288] are desirable (Scheme 20). Coupling of **49** to aldehyde **42** gives a derivative of (22*E*)-cholesta-5,22-dien-3β-ol (**9**), while a similar approach with **50** leads to a derivative of (22*E*)-ergosta-5,22-dien-3β-ol (**8**). Synthetic consideration of **50** suggests an approach from alcohol **51**. Such an approach has been reported for the enantiomer of **50**.[288] In that case, the C-24 epimer of **8** was synthesised by coupling to a C-22 steroidal aldehyde, suggesting this

approach to **8** to be viable. The desired enantiomer of alcohol **51** was prepared by the authors in a long sequence of steps involving an elegant chiral alkylation of enolates.[297, 298]

Consideration of alcohol **51** suggests a more facile preparation through side-chain cleavage of ergosterol at the C-22 double bond. Although oxidation clearly occurs, the Lemieux-von Rudloff oxidation of ergosterol (**4**) fails to give any isolable products. However, upon ozonolysis in pyridine and dichloromethane followed by reductive work-up with sodium *bis* (2-methoxy-ethoxy)aluminium hydride, alcohol **51** is isolable by steam distillation followed by preparative gas chromatography (Scheme 19).

Scheme 19.



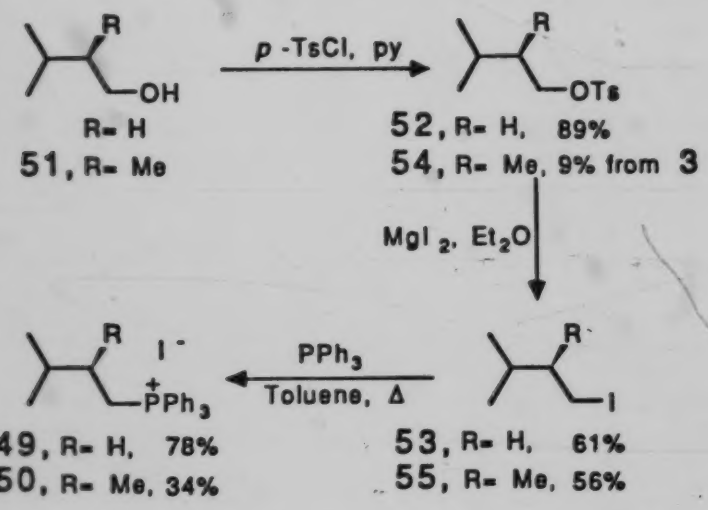
4 **51**

With a route to alcohol **51** available, a route from available 3-methyl-1-butanol to phosphonium salt **49**, as a model for the analogous reaction with **51**, was next sought. The 3-methyl-1-butanol reacts readily with *p*-toluenesulfonyl chloride to afford toluenesulfonate **52** (Scheme 20). Reaction of **52** with magnesium iodide in ether by the method of Place *et al*[299] then affords iodide **53**. Compound **53** readily undergoes substitution with triphenylphosphine, as shown by Bergmann and Dusza,[285] to give the desired triphenylphosphonium salt **49**.

Upon treatment of the crude mixture obtained from ozonolysis of ergosterol, which contains (2*R*)-2,3-dimethyl-1-butanol (**51**), with *p*-toluenesulfonyl chloride, the involatile toluenesulfonate **54** forms (Scheme 20). Separation from the other products of ozonolysis

is now facile. Reaction with magnesium iodide as for **52** gives highly unstable iodide **55**, which readily forms phosphonium salt **50**, provided that light and air are rigorously excluded from the reaction mixture.

Scheme 20.

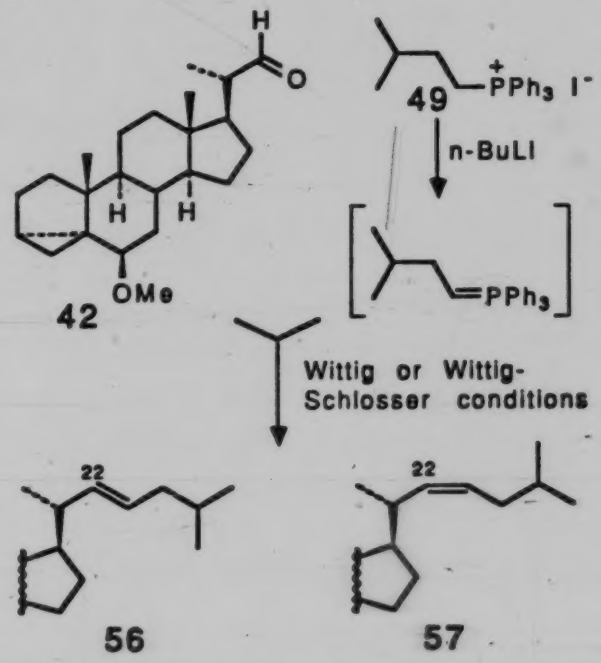


Although Wittig reaction of aldehyde **42** with phosphonium salt **49** proceeds smoothly (see below), one reaction with **50** under similar conditions failed to give the desired product: due to the somewhat poor yields in preparation of **50**, and development of the route to (22*E*)-ergosta-5,22-dien-3β-ol (**8**) described above, the latter reaction was not investigated further.

Wittig reaction of aldehyde **42** with phosphonium salt **49** gives a mixture of the desired (22*E*)- and (22*Z*)-isomers **56** and **57**, in modest yield (Scheme 21). Careful analysis of the carbon-13 NMR spectrum of the mixture, and subsequent isolation of the separate isomers (see below), shows that the two isomers are readily distinguished by the resonances of the carbons of the newly-formed double bond. Unfortunately, the proton NMR, although different for the two isomers, does not allow facile analysis of the composition of a mixture, since the proton resonances overlap considerably. This result

makes measurement of the coupling constant between the two protons, and hence assignment of *cis* and *trans* sterochemistry to the two isomers, difficult. Further, assigning the carbon-13 signals of the isomers is difficult. However, use of the ^{1}H-^{13}C heteronuclear shift correlation solves both problems. The signals for the two protons of each isomer are resolved into separate one-proton multiplets corresponding to each carbon signal. Examination of these multiplets shows that the most downfield carbon has an attached proton with coupling constants of 15 and 7 Hz, suggesting that this carbon signal is due to C-22 of the *trans* isomer **56**. The next most downfield carbon has an attached proton with coupling constants of 11 and 11 Hz, suggesting that this carbon signal is due to C-22 of the *cis* isomer **57**. In a similar fashion, the remaining two carbon signals can be assigned.

Scheme 21.



Unfortunately, the assigned values of the coupling constants between CH-22 and

CH-23 in the two isomers **56** (15 Hz) and **57** (11 Hz) do not distinguish them as rigorously as might be desirable. However, the assignments are consistent with subsequent experiments on the separated isomers: the infrared spectrum of **56** shows a clear peak at 965 cm^{-1}, which has been assigned to the *trans* isomer,[282] and both carbon-13[301] and proton[282, 283, 286] NMR chemical shifts are consistent with literature values for compounds related to the two isomers.

With the assignments for the two isomers, detailed examination of the influence of reaction conditions on the ratio of **56** to **57** is possible. Literature precedent suggested a considerable influence of solvent on this ratio, and therefore a study of variation of solvent on both yield and isomeric ratio of the isolated mixture of isomers was undertaken. The desire for predominantly *trans* stereochemistry also suggests investigation of the Schlosser modification[302, 303] of the Wittig reaction. The results of a series of experiments are summarised in Table 3.

Clearly, the Wittig-Schlosser reaction in ether affords the optimum ratio of *cis* to *trans* product. Surprisingly, the isomer ratio in THF is unaltered between conventional Wittig and Wittig-Schlosser reactions. One possible explanation is that the intermediate betaine, which is equilibrated between *erythro* (leading to *cis* olefin) and *threo* (leading to *trans* olefin) isomers via the β-oxido ylids, exists predominantly in the oxaphosphetane form in THF (Scheme 22). The oxaphosphetane may be less prone to further deprotonation than the β-oxido ylid.

An alternative Wittig approach to **56** involves use of a steroidal triphenylphosphonium salt, which condenses with a suitable aliphatic aldehyde. A route to phosphonium salt **58** (Scheme 25) has been reported.[289] This alternative approach might favour the *trans* product.

Reaction of alcohol **46** with *p*-toluenesulfonyl chloride gives toluenesulfonate **59**. Displacement of the toluenesulfonate with sodium iodide in acetone affords iodide **60** (Scheme 25).

Interestingly, when magnesium iodide in ether, which is very effective for conversion of aliphatic toluenesulfonates to iodides as described above, reacts with **59**, di-iodide **61** is the only observed product. Compound **61** probably derives from opening of the i-ether moiety of **59** or **61**, catalysed by magnesium iodide as a Lewis acid (Scheme 24).

Table 3.

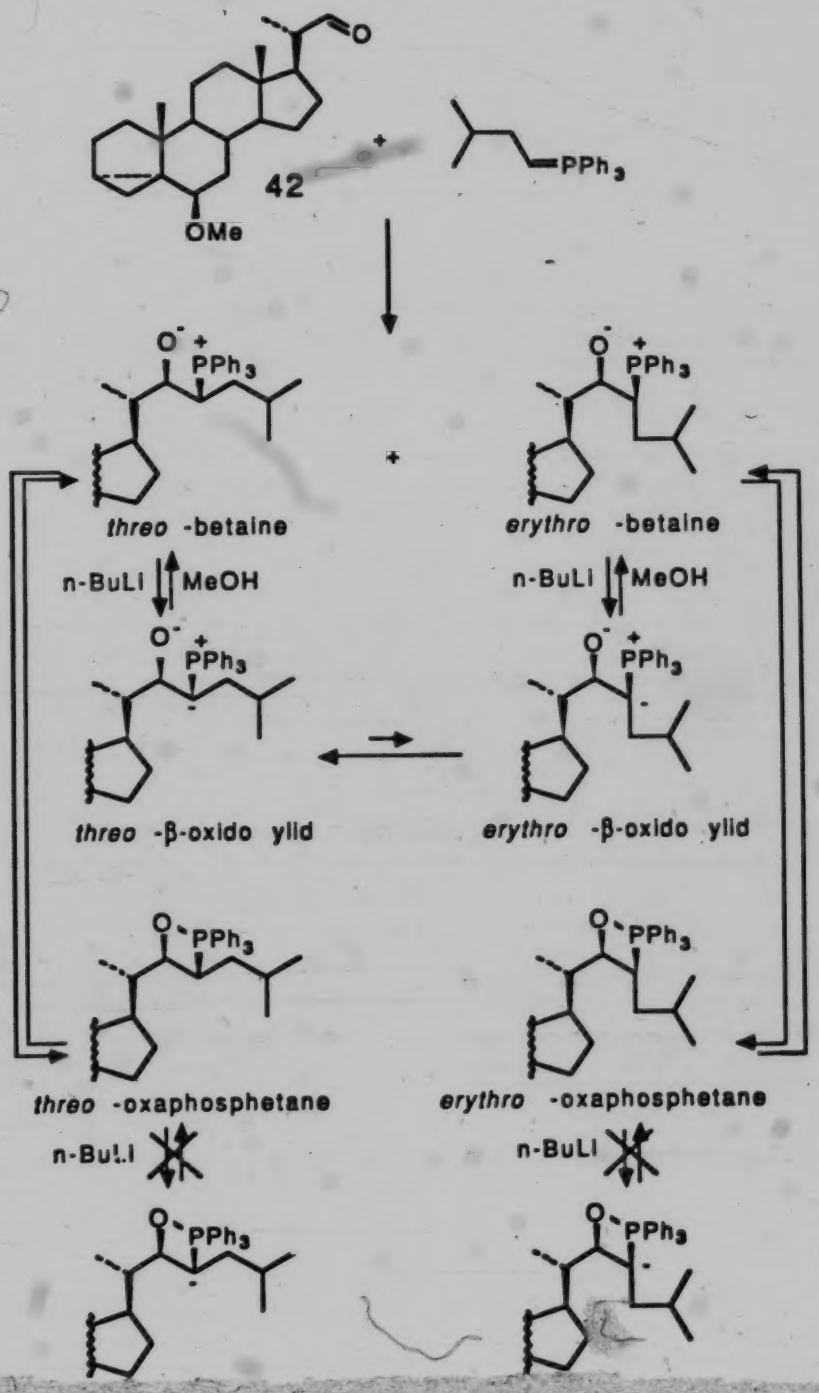
Ratio and yield of *cis* and *trans* isomers from Wittig and Wittig-Schlosser reactions between phosphonium salt **49** and aldehyde **42**

Reaction	Solvent	Temperature	Crude Yield (%)[c]	Ratio of **56** : **57**[d]
a	Hexane[g]	60 °C	9	≈50 : 50
a	THF	-78 °C[e]	47	12 : 88
b	THF	-78 °C[f, h]	13	13 : 87
b	THF	-78 °C[f]	53	13 : 87
a	Et_2O	25 °C[e]	32	51 : 49
b	Et_2O	25 °C[f]	54	56 : 44
b	Et_2O	-78 °C[f]	32	63 : 37
b	Et_2O	-78 °C[f, j]	28	75 : 25

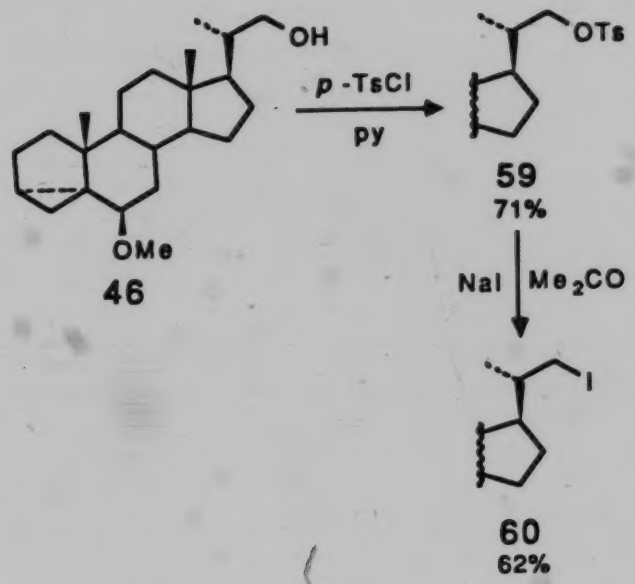
[a] Conventional Wittig reaction. [b] Wittig-Schlosser reaction. [c]Total yield of **56** and **57**. [d]Ratio from intensities of carbon-13 NMR signals. [e]Compound **49** was deprotonated with n-butyllithium at 25 °C; **42** was added at the stated temperature, and the mixture was then heated at reflux. [f]Compound **49** was deprotonated with n-butyllithium at 25 °C; **42** was added at the stated temperature, a second equivalent of n-butyllithium was added and, if at -78 °C, the mixture was warmed to -30 °C. One equivalent of methanol was added, and the mixture heated at reflux. [g]Following the procedure of Ref. 282. [h]Methyllithium was used in place of n-butyllithium. [j]Larger scale reaction.

Scheme 22.

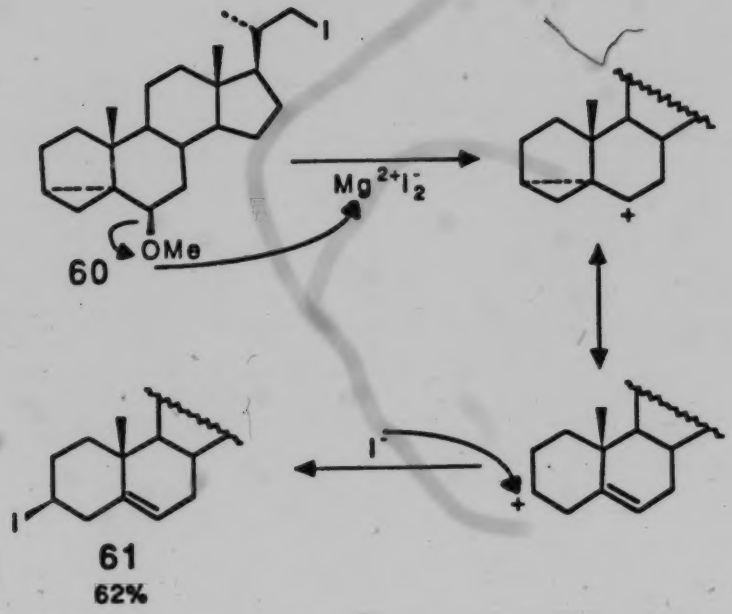
(Each *erythro-* or *threo-* isomer is a pair of diastereomers; only one diastereomer is shown for clarity)



Scheme 23.

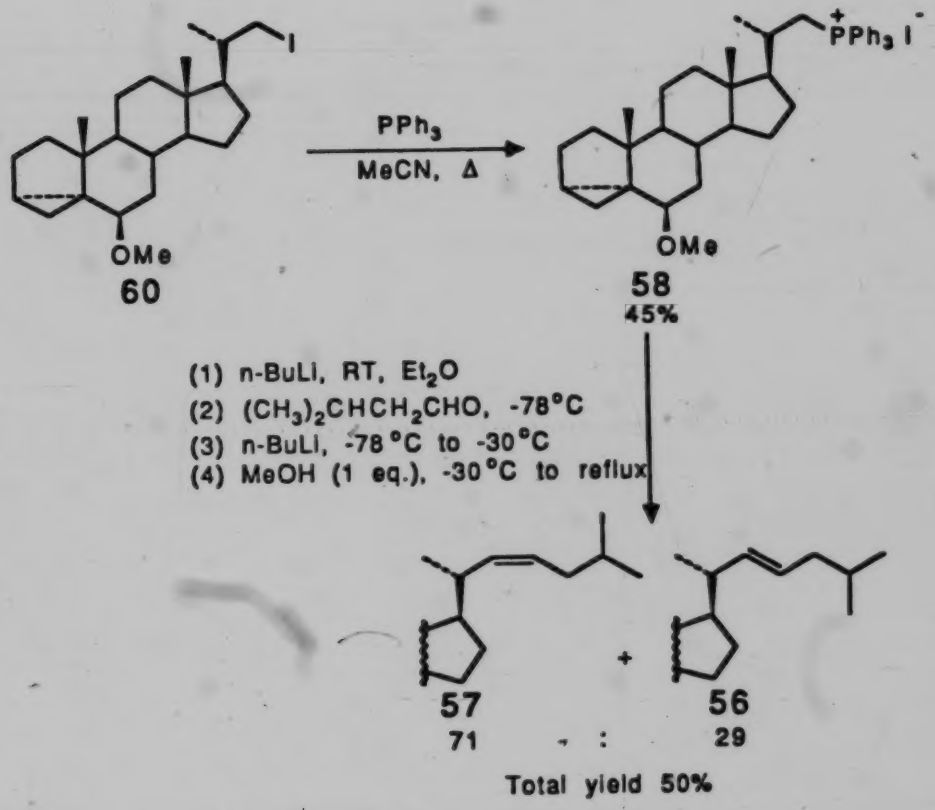


Scheme 24.



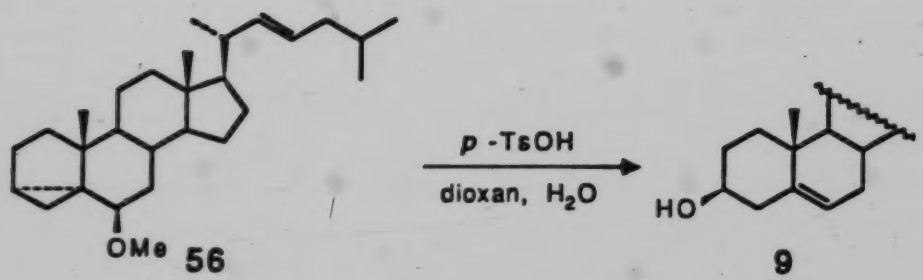
Iodide **60** undergoes substitution by triphenylphosphine to afford salt **58**. Reaction of **58** with 3-methylbutanal under Wittig-Schlosser conditions gives a mixture of **56** and **57** in a ratio of 29 : 71 (Scheme 25). Thus, optimum yields of *trans* isomer **56** are obtained from the steroidal aldehyde rather than the steroidal phosphonium salt.

Scheme 25.



Separation of **56** and **57** is quite facile by multiple chromatography over silver nitrate-impregnated alumina. In this fashion, **56** may be prepared containing only 2 - 3% of **57**, an unprecedented level of purity for derivatives of (22*E*)-cholesta-5,22-dien-3β-ol (**9**) prepared in this way. Treatment of **56** with *p*-toluenesulfonic acid in aqueous dioxan effects the i-ether to sterol rearrangement to afford the desired sterol **9** in 95% yield (Scheme 26).

Scheme 26.



56 **9** 95%

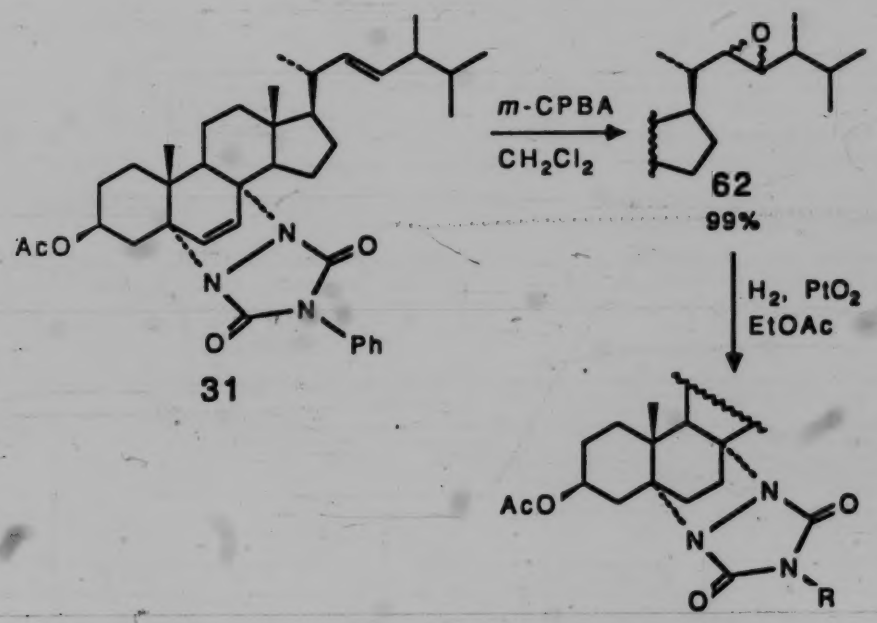
A similar Wittig-type reaction on a B-ring diene possessing a side-chain aldehyde functionality[253, 304, 305] in principle furnishes the last target sterol, (22*E*)-cholesta-5,7,22-trien-3β-ol (**7**).[287] One reported approach to synthesising such an aldehyde involves ozonolysis of ergosterol derivatives in which the B-ring has been protected as the N-phenyltriazoline-3,5-dione derivative.[253] Protection is necessary, since direct ozonolysis of ergosterol leads to rapid attack on the diene.[306] The authors report isolation of the side-chain aldehyde in 34% yield after oxidative work-up: presumably, the low yield is due to competitive ozonolysis of the remaining B-ring double bond.[307] Therefore, the possibility of a variation in this route was explored.

Ozonolysis of **31** followed by reductive work-up with sodium *bis* (2-methoxy-ethoxy)aluminium hydride fails to give any isolable products. However, although catalytic hydrogenation or ozonolysis causes reaction of the 6-7 double bond of derivatives like **31**, most electrophiles attack the side-chain double bond selectively. Thus, peracids afford only the side-chain epoxides **62**,[308] which in principle might be opened to afford the corresponding diols. These diols in turn could be cleaved to a derivatized side-chain aldehyde.[309]

Treatment of **31** with *meta*-chloroperoxybenzoic acid readily gives **62** as a mixture of (22*R*, 23*S*)- and (22*S*, 23*R*)-isomers (Scheme 27). The proposed structures are

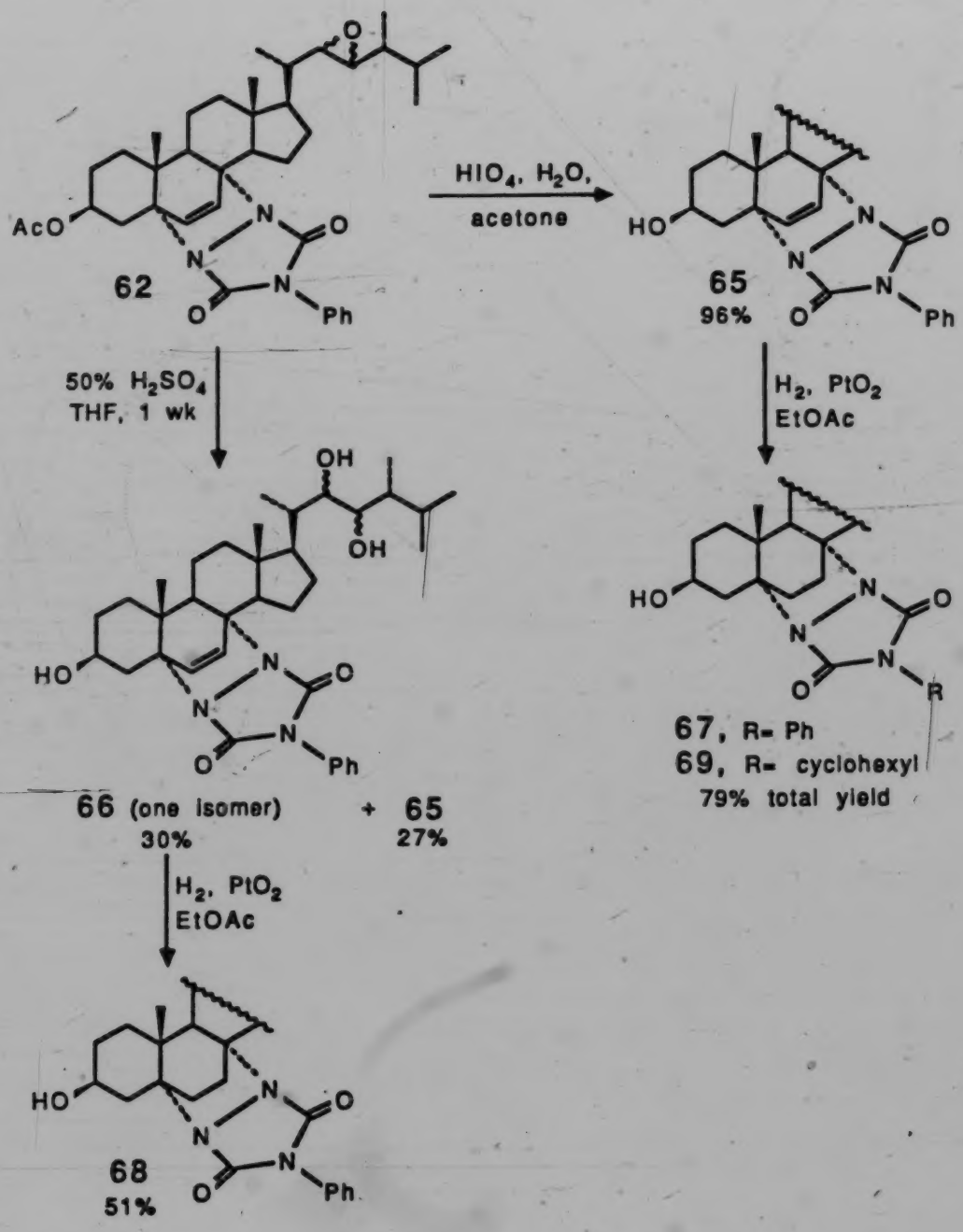
analogues **64**, in analogy to other N-phenyltriazoline-3,5-dione adducts described above. Interestingly, reaction of **62** with periodic acid in aqueous acetone leads only to cleavage of the 3β-acetate, to give 3β-sterols **65** (Scheme 28). No opening of the epoxide occurs, despite use of quite drastic conditions. In contrast, use of perchloric acid in aqueous THF affords a small yield of triol **66**, along with **65**. Use of 50% sulfuric acid in THF for 1 week allows up to half of the material to be converted to **66**, while half is converted to **65**. Surprisingly, the resulting triol **66** is a single compound by carbon-13 NMR. This unexpected result suggests that only one stereoisomer of the epoxides is undergoing reaction. Although the stereochemistry of the diol is not determined, this observation may be of interest in relation to synthetic approaches to brassinolides, which possess a 22, 23-diol moiety.[260-262]

Scheme 27.



63, R= Ph
64, R= cyclohexyl
93% total yield

Scheme 28.


AcO
62
HIO_4, H_2O,
acetone
HO
65
96%
Ph
50% H_2SO_4
THF, 1 wk
H_2, PtO_2
EtOAc
OH
OH
HO
66 (one isomer)
30%
+ 65
27%
67, R= Ph
69, R= cyclohexyl
79% total yield
R
H_2, PtO_2
EtOAc
HO
68
51%

Epoxides **65** and triol **66** form dihydro derivatives **67** and **68**, respectively, upon catalytic hydrogenation for characterisation by high resolution mass spectrometry. In the case of **65**, some N-cyclohexyl analogue **69** is also formed.

The low yield associated with the opening of the epoxide, due to the inability to convert half of the material and to losses in isolation, coupled with the requirement for reprotection of the 3β-hydroxyl group prior to Wittig reaction, compelled rejection of this approach. Reaction of **31** with osmium tetroxide,[310] in an attempt to form the side-chain diol directly,[311] also failed to give any pure products.

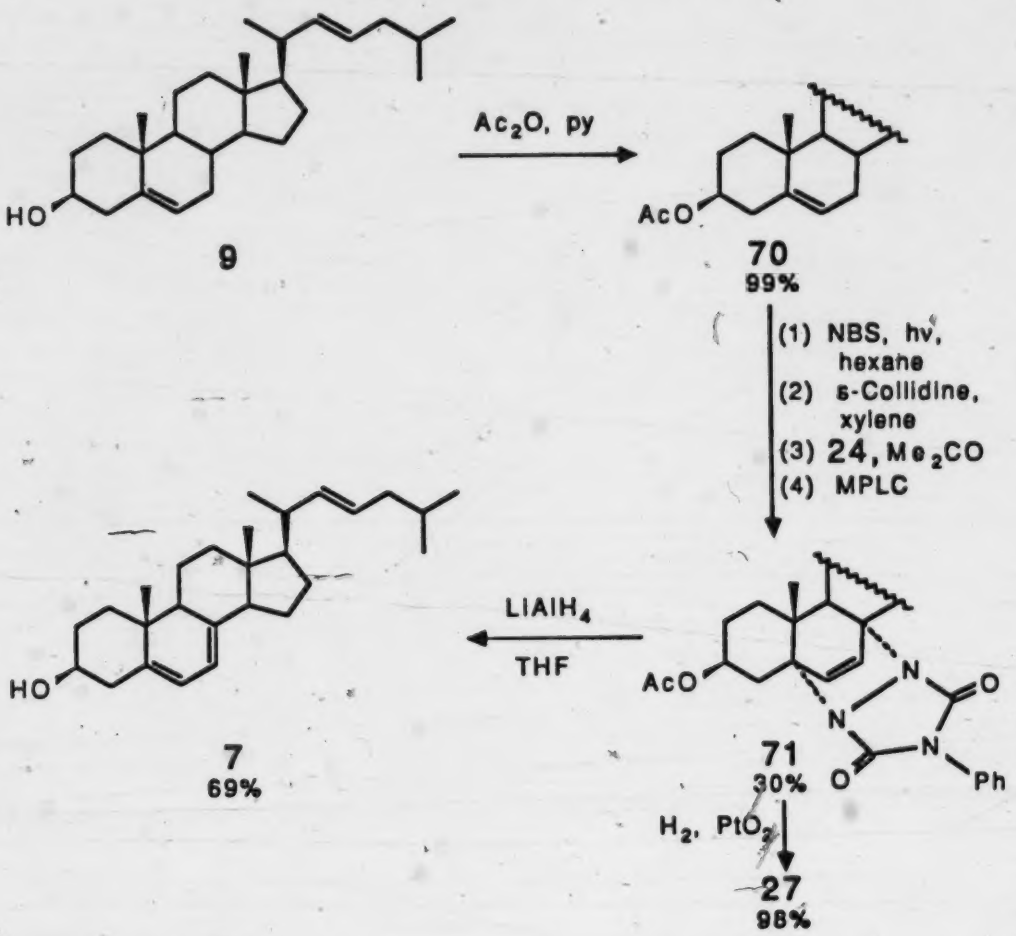
An alternative approach to (22*E*)-cholesta-5,7,22-trien-3β-ol (**7**) that has been reported[287] involves introduction of the B-ring diene into (22*E*)-cholesta-5,22-dien-3β-ol (**9**) by benzoylation and allylic bromination followed by dehydrobromination. The yield was 20%. Following the successes of this approach described earlier, isolation of the N-phenyltriazoline-3,5-dione adduct possessed potential to lead to the desired product in higher yield.

The precursor acetate **70** forms readily upon acetylation of **9** (Scheme 29). Allylic bromination at C-7 with N-bromosuccinimide, followed by elimination with *s*-collidine, affords a mixture of sterol acetates that react with N-phenyltriazoline-3,5-dione (**24**) to give a mixture of adducts. In this case, presumably, some bromination occurs at the allylic positions in the side-chain, leading to side-chain dienes which react with **24** to form adducts. However, the mixture of adducts separates by HPLC or on a medium-pressure liquid chromatography column to afford adduct **71** in 30% yield. Upon catalytic hydrogenation, adduct **71** gives dihydro analogue **27**, which is identical to material obtained from **26**. The other adducts from the reaction elute from the column as mixtures of compounds. Adduct **71** is readily converted to the desired sterol **7** by treatment with lithium aluminium hydride. Compound **7** is surprisingly unstable, and therefore the small amounts obtained were characterised only by proton and carbon NMR before

Sterol **7** is also a natural product

Tetrahymena pyriformis .312, 313

Scheme 29.

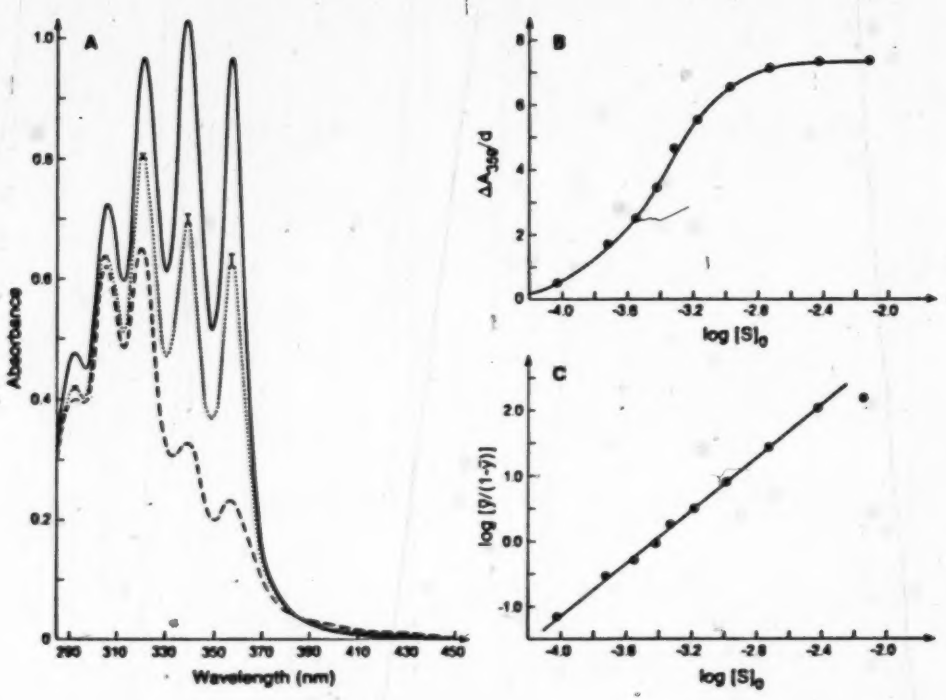


Studies on Polyene-Sterol Binding

Once the synthetic sterols are available, investigation of the influence of sterol structure on binding to polyenes can be undertaken. The simplest approach appears to be examination of the equilibrium constants of binding by ultraviolet (UV) spectroscopy in aqueous suspension. This approach appears most attractive, because the unknown variables that occur in experiments with whole cells or vesicles are eliminated.

Preliminary experiments with fungichromin (1) show that binding to cholesterol (3) and ergosterol (4) occurs when solutions of these sterols in THF are added to a solution of 1 in 10% THF/water. However, in a solvent mixture of 30% THF/water, no binding occurs. This effect is in general agreement with literature results,[89] which show a rapid decrease in binding as the amount of organic solvent increases. As a compromise, the solvent 10% THF/water can be used, since, as is not the case in pure water, 1 obeys the Beer-Lambert law in this solvent over the concentration range of interest. This situation, indicative of monomeric 1, is desirable as it facilitates analysis. However, binding may be stronger in water alone. By stepwise addition of aliquots of sterols, a binding curve may be constructed. For mathematical analysis, the absorbance at 356 nm was used. This parameter has been argued[111] to reflect binding better than the ratio of absorbances at 356 nm and 320 nm used in other studies for filipin.[89] The curve does not fit a normal Scatchard plot,[314, 315] in contrast to the results of Bittman and coworkers in vesicle sudies with amphotericin B.[118, 131] In that case, the authors report a correlation over a limited region of the Scatchard plot. Further analysis of the binding curve for cholesterol, however, shows a good correlation when the data is analysed on a Hill plot.[315-317] Figure 17a shows some ultraviolet absorbance data from a binding experiment. The spectra are of fungichromin (1). The upper plot shows 1 alone, the middle spectrum has 1 46% bound to cholesterol (3), and the lower plot is of 1 with a large excess of 3 present so that it is fully bound. The full binding data is plotted as a binding curve in Figure 17b, and on a

Figure 17. (a) UV spectra of 330 μM fungichromin (1) in 10% THF/ H_2O:——— , in absence of cholesterol (3); ..... , with 377 μM 3; error bars represent the total change in absorbance between the first and final measurements. and -----, with a large excess (7.3 mM) of 3. (b) Binding curve for the complete data from (a). (c) Hill plot. For explanation of symbols, see experimental section.



Hill plot in Figure 17c. The apparent equilibrium binding constant, K_{app}, and the cooperativity, n, can thus be determined: the slope of the plot is n, and the intercept is $n\log K_{app}$. For cholesterol (3), $n = 2.00 \pm 0.07$ and $K_{app} = (2.50 \pm 0.15) \times 10^3\ M^{-1}$. Errors were determined by linear regression: similar errors were found in the other sterols that were fitted to Hill plots. One interpretation of the cooperativity of 2.0 is that though there are two binding sites on the polyene, in free polyene only one site may bind. However, once that first site is occupied, the second site becomes available, and binds another molecule of sterol strongly.[315]

This data analysis also holds for some of the other sterols and analogues tested (see later), but does not hold true for ergosterol (4). For 4, the Hill plot has variable slope. Another approach to evaluation of apparent binding constants measures the amount of sterol required to obtain half of the maximum change in ultraviolet absorbance,[89] which is equal to $1/K_{app}$. Analysis in this fashion shows that ergosterol (4) has $K_{app} = (3.5 \pm 0.2) \times 10^3\ M^{-1}$, or about 1.5 times that of cholesterol (3), which is in reasonable agreement with literature observations.

With a small but significant difference between ergosterol (4) and cholesterol (3) observed, binding curves for fungichromin (1) and the synthetic sterols 6, 8, 9, 21 and 22 can usefully be determined. However, no systematic trend that can be related to sterol structure can be seen. Clearly, the small differences in the binding curves are insufficient to allow definitive analysis of the influence of sterol structure on binding, partly because the approach to equilibrium binding is quite slow close to the midpoint of the binding curve. Allowance for this effect is made during the experiment by waiting until no further changes in absorbance are observed. However, the effect may introduce considerable error in the binding constant. This kinetic factor in polyene-sterol binding has been investigated extensively in the literature.[118, 128, 129, 132] In the present work, no obvious correlation is seen between structure and speed of approach to equilibrium. The experiment also suffers from potential errors due to insolubility of the sterol, the changing concentration of

THF (typically from 10 to 14%), and accurate measurement of $\hat{y}$ when 1 is mostly free or mostly bound.

Similar experiments with amphotericin B reveal much stronger binding to sterols: binding occurs in 25% THF/water, under which conditions K_{app} for cholesterol is (3.3 ± 0.2) X 10^4 M^{-1}, or approximately 13 times larger than that for fungichromin in 10% THF/water. The binding process is again cooperative, giving a linear Hill plot for cholesterol (n = 1.4 ± 0.1) but not for ergosterol. Ergosterol, however, also only has K_{app} = (5.9 ± 0.3) X 10^4 M^{-1}, or about 1.8 times K_{app} for cholesterol. This difference was again considered to be insufficient to allow definitive analysis of binding to the synthetic sterols.

Greater differences in the effects of different sterols might be observed in a biological assay. One such approach that has been reported uses *Acholeplasma laidlawii*, an organism that grows without biosynthesis of sterols. However, addition of sterol[318, 319] and fatty acids[320] to the growth medium causes incorporation into the cell membrane. Therefore, cells grown in the presence of cholesterol show greater sensitivity to polyene antibiotics than those grown without sterol.[114, 126, 151] Studies in collaboration with Professor R. N. McElhaney (Department of Biochemistry, University of Alberta) are in progress, directed towards incorporation of the synthetic sterols into *Acholeplasma laidlawii* for investigation of growth inhibition and potassium release.

For comparison with the binding data, Professor S. Fraga (Department of Chemistry, University of Alberta) has obtained theoretical calculations[321] of the interaction energy[322] of amphotericin B with cholesterol. In order to extend this approach to the synthetic sterols, the atomic coordinates for the sterols are required. For cholesterol (3)[323] and ergosterol (4),[324] this data is available in the literature from X-ray crystallography. However, X-ray crystal structures have not been reported on the synthetic sterols: therefore, attempts were made to crystallise them, and obtain X-ray data.

Ergost-5-en-3β-ol (22) was chosen as the first sterol. Of the literature methods for

crystallisation of sterols[323, 325-327] and (using **40**) sterol acetates[327] investigated, the method of Nordman and coworkers[325] proves most effective in this case. However, although an X-ray diffraction pattern is obtained, it is not solvable using standard techniques. Like other sterols, **22** crystallises with four or more molecules per unit cell,[323, 325] which gives too many atoms in the unit cell to obtain an exact solution. In collaboration with Professor Nordman (University of Michigan, Ann Arbor), the structure solution of **22** is under investigation, using advanced computational methods developed for analysis of complex molecules.

With the full assignment of the proton and carbon-13 NMR spectra of fungichromin (**1**) known, the effect of complexing by sterol might be investigated by NMR, which could give direct information on the relative molecular orientation of polyene and sterol molecules in the complex. However, because the complex does not form in systems containing large amounts of organic solvent,[89] and because the sterols are poorly soluble in aqueous media, NMR experiments on mixtures of polyenes and sterols must be done in a non-homogeneous state. Early experiments used proton NMR of vesicles.[140] Deuterium NMR has also been used on vesicles containing deuterated sterol[146] or lipid.[143] A similar approach by ESR using spin-labelled steroids[130, 145, 148] or lipids[130, 144, 145, 147, 149, 156] has also been reported, as have studies of the changes in the proton and sodium-23 NMR spectra of nystatin binding to sodium ions.[60] Pierce *et al*[157] used phosphorus-31 NMR to study permeability changes of vesicles with or without sterol upon binding to nystatin. No direct structural information on the sterol-polyene complex geometry is available from this work, however.

The availability of **1** labelled at most positions with deuterium from the biosynthetic studies prompted investigation of binding by deuterium NMR. In collaboration with Dr. Ian Smith (National Research Council, Ottawa), the deuterium NMR spectra of this material in vesicles with or without sterol[143, 146] is being investigated.

Another approach to the use of NMR to investigate binding, which overcomes the

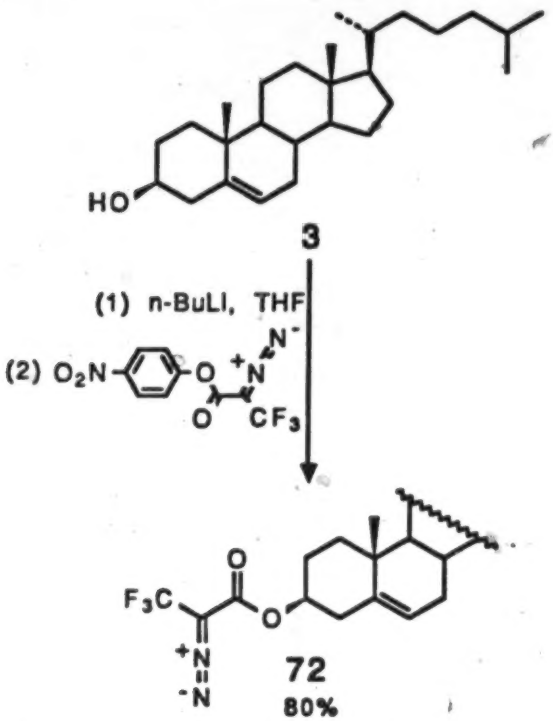
problems associated with solubility of the components during the NMR experiment, involves photoaffinity labelling.[328] If a modified sterol possessing a photoactivatable functionality is bound to the polyene, and the complex photolysed, the resulting reactive intermediate on the sterol may react with the polyene to form a new covalent bond. Without the polyene, the intermediate reacts by rearrangement, or with the solvent. After isolation, structural analysis of the covalent adduct in the solvent of choice could reveal the site of attachment of the sterol to the polyene. The site of attachment may reflect the proximity of the two points of attachment in the initially-formed complex.

A wide variety of photoaffinity labels,[328, 329] and many labelled steroids,[329-336] have been reported. In some cases, the natural α,β-unsaturated ketone of certain steroids has been used for photoaffinity labelling.[335-339] One of the most attractive photoactivatable groups is the 2-diazo-3,3,3-trifluoropropionyl functionality,[340, 341] which has been attached to spermidine and spermine,[342] sugars,[343, 344] phospholipids,[345-348] β-naphthol,[349] retinal,[350] chymotrypsin[341] and hormones.[351] Use of this group has several advantages. First, a carbene is generated upon photolysis. Carbenes are well known to be less selective than nitrenes, which are the other commonly used species. Such selectivity is desirable, since the carbene then undergoes reaction with the molecule to be labelled at the closest site, in preference to attack at the most reactive site.[341] Second, Wolff rearrangement, often a major side-reaction in carbenes, does not occur to as great an extent, due to stabilisation by the trifluoromethyl group.[341] Third, the trifluoromethyl group allows fluorine-19 NMR to be used as a probe for new compounds in the complex mixture of products. The high sensitivity, large chemical shift dispersion, and lack of interference in fluorine-19 NMR make this very attractive. The location of fluorine close to the reactive site makes the chemical shift highly sensitive to the nature of the reaction products. Finally, the group is stable to aqueous HCl.[341] This stability allows synthetic manipulation during preparation of the desired sterols.

First, the photoaffinity labelled steroid 3β-(2'-diazo-3',3',3'-trifluoropropionyl-

oxy)-cholest-5-ene (**72**) was prepared. Reaction of cholesterol (**3**) with n-butyllithium to generate the alkoxide anion, followed by addition of *p* -nitrophenyl 2-diazo-3,3,3-trifluoropropionate, gives **72** in 80% yield (Scheme 30).

Scheme 30.



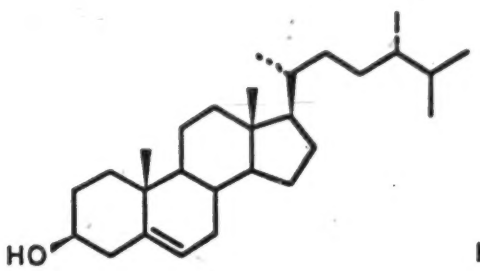
Determination of the binding constant of **72** to fungichromin (**1**) shows that this compound binds only very weakly, if at all, to the polyene: $K_{app} \leq 10\ M^{-1}$. This result is somewhat surprising, since literature reports indicate that cholesterol acetate (**5**) binds to polyenes, albeit more weakly than cholesterol (**3**) itself.[89] Further studies show that, in 10% THF/water, acetate **5** binds to **1**; however, **5** has apparent binding constant $K_{app} = 460 \pm 50\ M^{-1}$ and a cooperativity $n = 1.02 \pm 0.05$, as compared to cholesterol (**3**), for which $K_{app} = 2500 \pm 150\ M^{-1}$ and $n = 2.00 \pm 0.07$. This result suggests that the presence of the acetate group in **5** diminishes the ease of binding of the first molecule of sterol, and completely prevents the cooperative binding of a second molecule. Clearly, the larger ester

group in **72** as compared to **5** further diminishes the binding. Although other explanations are available, the results suggest steric interference of binding by groups on the oxygen atom of cholesterol.

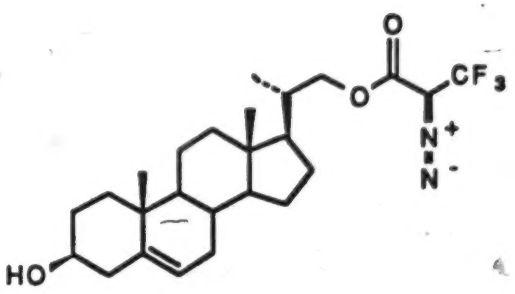
Despite the lack of binding, the synthetically readily available **72** proved useful in preliminary investigations of the proposed labelling process. Compound **72** absorbs strongly at 237 nm. Experiments on a suspension of **72** in 10% THF/water show that photolysis in a quartz tube by a medium-pressure mercury lamp leads to a variety of products, as shown by fluorine-19 NMR. The half-life for photolysis (ca. 2 - 3 hr) can be readily determined by dissolving aliquots of the suspension in THF, and by following the decrease in absorbance at 237 nm by ultraviolet spectroscopy. The same experiment on a control shows that a slow (half-life ca. 24 hr) spontaneous decomposition occurs.

When fungichromin (**1**) is photolysed under the same conditions, rapid decomposition occurs.[352] The half-life is of the order of 3 - 4 min, which clearly makes the photoaffinity labelling experiment impossible. However, using a diffraction filter which transmits the maximum amount of light at ca. 235 nm between the lamp and the reaction vessel essentially prevents decomposition of the polyene, which absorbs above 300 nm, as shown by UV spectroscopy and re-isolation of **1**.

The chosen photoaffinity label is also attractive because it can be condensed with a side-chain steroidal alcohol. Derivative **46** is an ideal candidate. The product may be converted to **73** which is potentially approximately iso-steric with ergost-5-en-3β-ol (**22**).



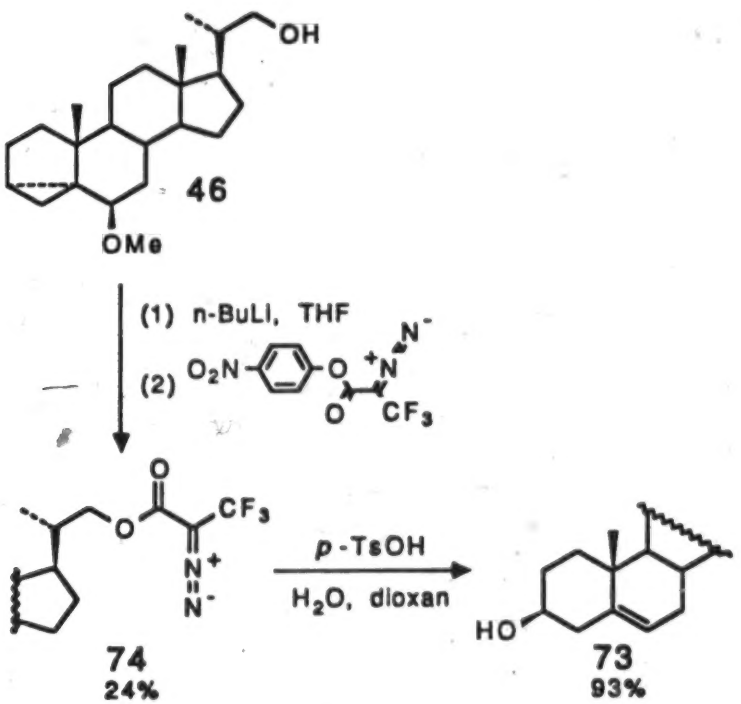
22



73

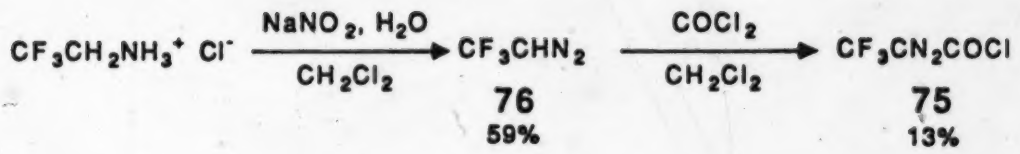
Treatment of alcohol **46** with n-butyllithium, followed by addition of *p*-nitrophenyl 2-diazo-3,3,3-trifluoropropionate, gives **74** in 24% yield (Scheme 31). Despite several variations of conditions, and the use of N, N-dimethylaminopyridine as base instead of n-butyllithium, the yield is never higher. One possible explanation for the low yield is that reaction of the alkoxide anion of **46** with the diazo-ester is slow by comparison with that of the cholesterol anion. The remaining base may then destroy the photoaffinity label.

Scheme 31.



In order to circumvent this problem, the corresponding acid chloride, a more reactive acylating agent, could perhaps be used. Acid chloride **75** was prepared, albeit in poor yield, following the method of Chowdhry *et al.*[341] Trifluorodiazoethane (**76**) is readily prepared by reaction of trifluoroethylamine hydrochloride with sodium nitrite (Scheme 32).[353] Reaction of **76** with phosgene affords **75**.

Scheme 32.



Condensation of alcohol **46** with acid chloride **75** using either deprotonation with n-butyllithium, or N, N-dimethylaminopyridine as base, failed to give improved yields of **74**. However, the efficient conversion of **74** to **73** under acid catalysis by *p*-toluenesulfonic acid[267] (Scheme 31) compensates somewhat for the poor yield in the first step.

A binding curve for **73** clearly shows efficient binding to fungichromin (**1**). The data fits the Hill plot, giving a binding constant $K_{app} = (2.5 \pm 0.15) \times 10^3\ M^{-1}$, and a cooperativity $n = 2.6 \pm 0.1$ (compare with cholesterol, $K_{app} = (2.5 \pm 0.15) \times 10^3\ M^{-1}$, $n = 2.00 \pm 0.07$): **73** binds as strongly to fungichromin as the natural sterol, but with higher cooperativity. However, upon performance of the photoaffinity labelling experiment, no new labelled adduct can be detected by TLC or proton or fluorine-19 NMR. The results do, however, clearly show that considerable photolysis of the diazo-ester occurs to give a product distribution similar to that obtained upon photolysis of **73** alone.

One possible explanation for the lack of labelling of the polyene by the photoaffinity-labelled sterol, despite the implied formation of the carbene, is that the sterol side-chain is not in close proximity to the polyene in the complex. However, the cooperativity for **73** binding to fungichromin (**1**) is larger than that for cholesterol (**3**), suggesting that the side-chain structure influences binding. Possibly, the side-chain in cholesterol reduces the effectiveness of binding due to unfavorable steric effects, while a different conformation of the side-chain in analogue **73** reduces or eliminates these effects because the side-chain is no longer close to the polyene. Conceivably, the difference in conformation arises from different effects in solvation of the more polar side-chain in **73**

and the lipophilic side-chain of **3**. Sterol side-chain length has been shown to influence binding of polyenes.[129, 132, 174] Sterols without a side-chain (e. g. androstene-3β-ol) do not bind in the cases cited.[174]

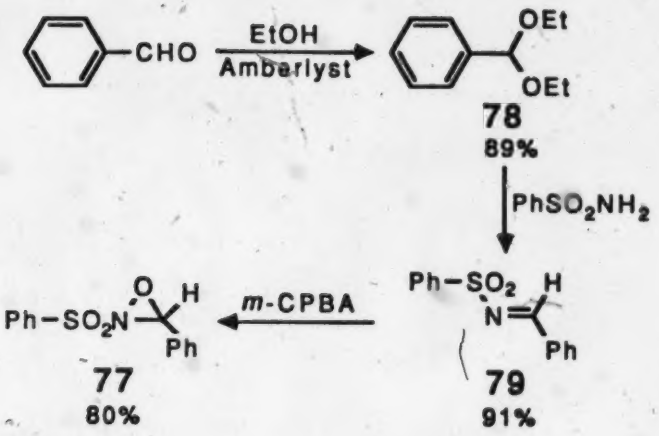
Considerable evidence in the literature shows that binding is more favorable for sterols possessing a 5,7-diene (e. g. ergosterol (**4**)) than for those with a single double bond at the 5-6 or 6-7 positions (e. g. cholesterol (**3**)) (see introduction). This effect has been generally accepted as indicating that a flatter, more planar structure, especially in the B-ring of the sterol, favors binding. Clearly, this favorable binding indicates that the B-ring is in close proximity to the polyene in the complex, suggesting that location of a photoaffinity label as a substituent in this area of the sterol might be desirable. A suitable compound could be produced by acylation of a hydroxyl group in a hydroxylated sterol.

Oxidation of the enolate of cholest-4-en-3-one, using methods developed in this[354] and other[355, 356] laboratories, could possibly lead to introduction of a hydroxyl group into the sterol, hopefully at C-2 or C-4 rather than C-6. Literature precedent[357] suggests that the kinetically favored enolate forms by deprotonation at C-2, while the thermodynamically favored enolate forms at C-6. For the oxidation, the method of Davis *et al*,[356] using oxaziridine **77**,[355, 358] was selected.

Reaction of benzaldehyde with ethanol, catalysed by cation exchange resin, gives benzaldehyde diethyl acetal (**78**) (Scheme 33).[359] Upon heating of **78** with benzenesulfonamide, condensation occurs to give imine **79**.[356] Epoxidation of **79** with *meta*-chloroperoxybenzoic acid then affords the desired reagent **77**.[355, 358]

Formation of the enolate(s) of cholest-4-en-3-one at -78 °C with lithium diisopropylamide, followed by addition of **77**, gives mainly recovered starting material. However, extensive purification affords 8% of a hydroxylated sterol that is ca. 90% pure. In the proton NMR spectrum, the olefinic proton appears as a sharp singlet, indicating that the product is not the C-4 hydroxylated compound, since it would possess an alkene proton at C-6 which would show coupling to H-7. The proton on the carbon bearing the hydroxyl

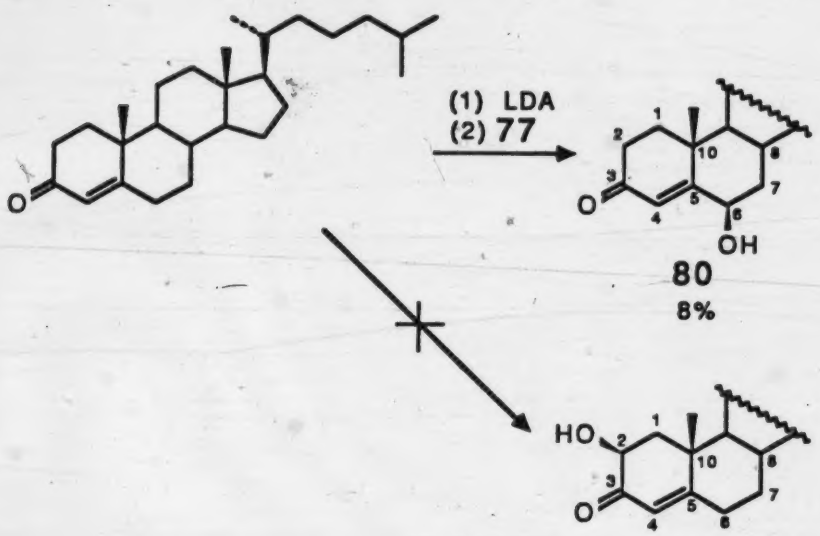
Scheme 33.



group is a doublet of doublets, J = 3 and 3 Hz. These two equal and small couplings show that the proton is equatorial, and thus, surprisingly, that oxidation occurs on the β-face of the sterol. If the proton were axial, a large coupling would be expected, since one adjacent proton must be at a dihedral angle close to 180°. However, these results do not allow distinction between hydroxylation at C-2 or C-6. The structure is readily solved from the proton-proton COSY spectrum of the material. Examination of the spectrum reveals that the proton on the carbon bearing the hydroxyl group couples to two protons at chemical shifts of δ 2.03 and δ 1.26. These two protons must be either the protons on C-1, in the C-2 hydroxylated compound, or those on C-7, in the C-6 hydroxylated compound. Close examination of the COSY spectrum shows that the signal at δ 1.26 is a doublet of doublets, J = ca. 15 and 15 Hz. One of these coupling constants is the geminal coupling constant to the other proton on the same carbon atom. The small coupling (3 Hz) to the proton on the hydroxyl-bearing carbon is not observed due to the limit of resolution. The residual large coupling constant remains to be explained. Explanation is difficult if the signal at δ 1.26 is due to a proton at C-1 in the C-2 hydroxylated compound, since C-1 is adjacent to C-10, a quaternary centre. However, if the signal is due to a proton at C-7 in the C-6 hydroxylated compound, the residual coupling can be explained readily: it is due to coupling to the

proton at C-8. These experiments thus show that the product is 6β-hydroxycholest-4-en-3-one (80) (Scheme 34).

Scheme 34.



Unfortunately, 80 is the only regioisomer that is not suitable for synthesis of a photoaffinity analogue, because the double bond is now 'fixed' in the wrong position. A further problem with this approach becomes apparent when the photoaffinity-labelled ester of cholesterol 72 is treated with sodium borohydride in methanol, as a model for the subsequent necessary reduction of the ketone moiety in photoaffinity-labelled derivatives of hydroxycholestenones. Rapid decomposition of the diazo-ester moiety of 72 occurs upon the treatment with borohydride. Fluorine NMR indicates a mixture of products, which is not readily separable by chromatography. The problems assoeiated with this approach . prompt investigation of a different scheme. An alternative synthetic route to a sterol with a photoaffinity label substituent close to the B-ring involves substitution at C-7. Reduction of a C-3 protected sterol with a ketone at C-7 could in principle give a mixture of epimeric alcohols, which could then be used to attach the label. In order to determine which of the two epimers binds most effectively to fungichromin (1), the C-7 acetates can be used as

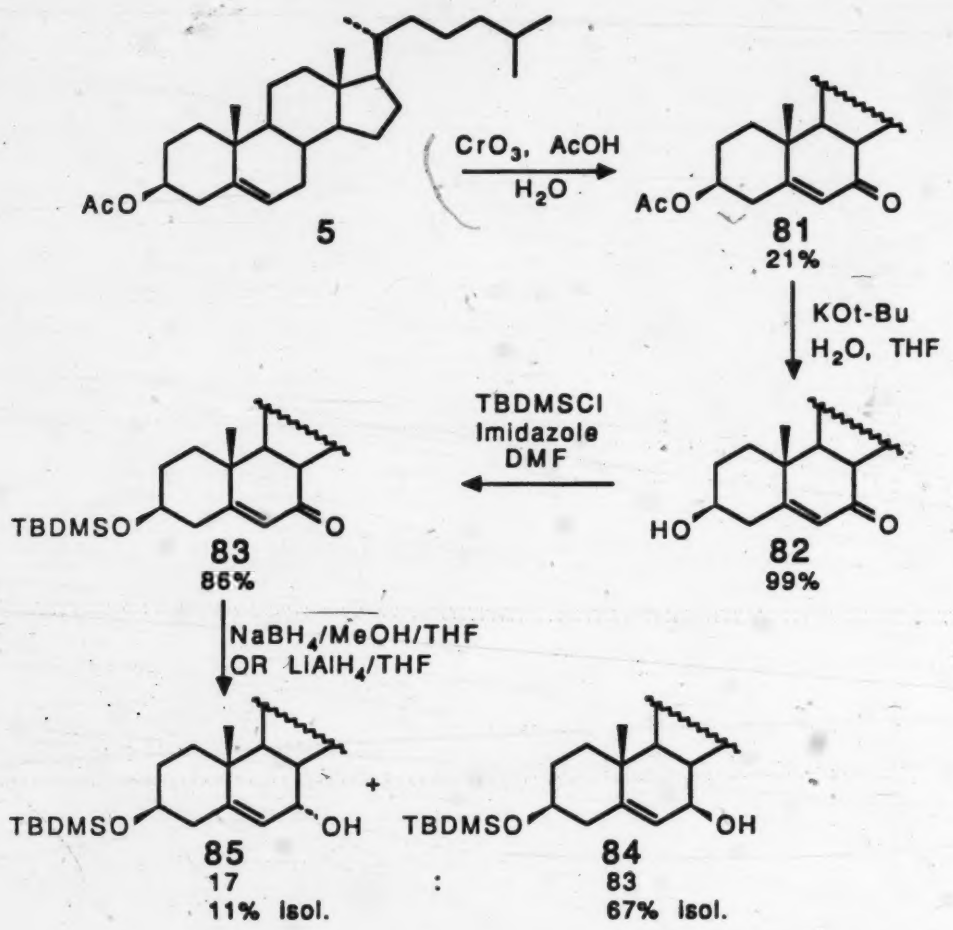
models.

Oxidation of cholesterol acetate (**5**) with chromium trioxide in aqueous acetic acid according to the method of Dauben and Fonken[360] gives 7-oxocholesterol acetate (**81**)[240, 360] in 21% yield (Scheme 35). Deprotection at C-3 with potassium *t*-butoxide in aqueous THF affords 7-oxocholesterol (**82**)[361] quantitatively. Sterol **82** is readily protected as the *tert*-butyldimethylsilyl ether **83** with *tert*-butyldimethylsilyl chloride and imidazole in DMF.[362] Ketone **83** gives a mixture of α- and β-alcohols **84** and **85** upon reduction with sodium borohydride in methanol/THF or lithium aluminium hydride in THF.

This mixture is readily separated by chromatography, which is in marked contrast to separation of the mixture of alcohols produced when acetate **81** is reduced. In contrast to the reduction of 7-oxocholesterols to equal amounts of both isomers, as described in literature reports,[363, 364] reduction in this fashion affords the equatorial 7β-hydroxy-3β-(*tert*-butyldimethylsilyloxy)cholest-5-ene (**84**) in preference to the 7α isomer **85**. The ratio of isomers, determined by NMR, is 83 : 17 with both borohydride and lithium aluminium hydride. This ratio reflects the expected preference for attack on the α-face of the sterol.

At this point, an alternative approach to investigation of the mode of binding of sterols to polyenes becomes apparent. If fluorine were introduced into the sterol, examination of the fluorine-19 NMR of sterol in vesicles with and without polyene might show differences in chemical shifts and coupling constants that depend on the location of the fluorine. Furthermore, heteronuclear nuclear Overhauser effects could perhaps be observed between the polyene protons and the sterol fluorine, allowing direct determination of points of proximity between the two molecules. The use of fluorine-19 NMR is attractive for the same reasons as those that applied in the photoaffinity labelling experiment; introduction of fluorine at C-7 appears ideal for the same reasons as those that prompted the introduction of a photoaffinity label at that site.

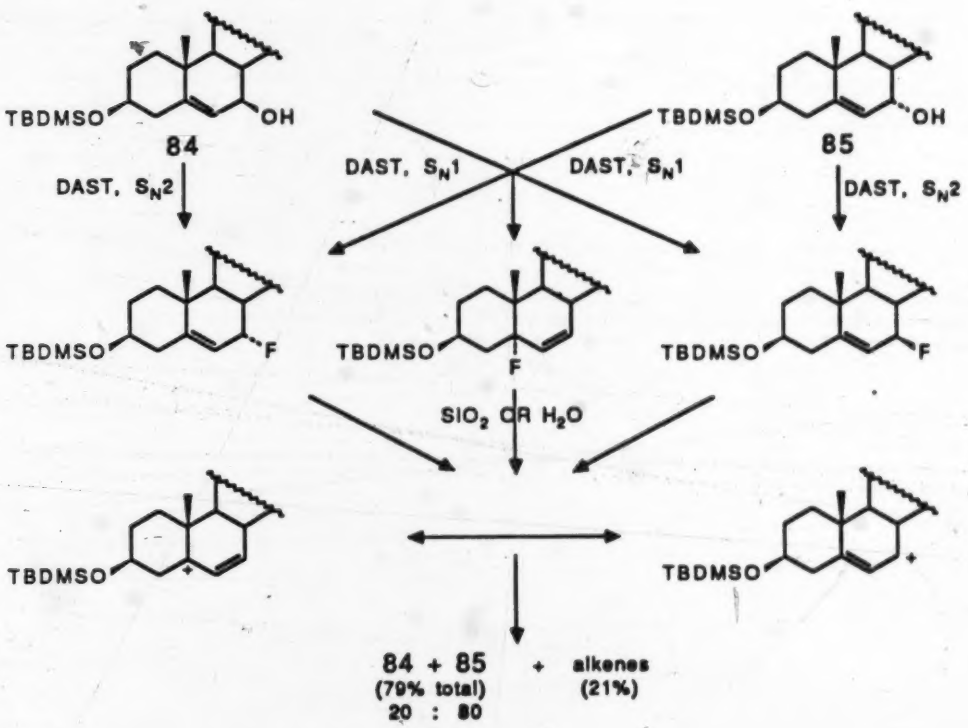
Scheme 35.



Remarkably, treatment of ketone acetate **81** with diethylaminosulfur trifluoride (DAST)[365, 366] fails to give any reaction. However, the reduced alcohols **84** and **85** do react readily with DAST to afford a mixture of *three* fluorides, as shown by fluorine-19 NMR of the crude reaction mixture. Interestingly, aqueous work-up, silica gel chromatography, or simply stirring with silica gel for a short time (3 - 5 min), causes complete decomposition of the fluorides. The product is a mixture of alcohols, **84** and **85**, in a ratio of ca. 20 : 80. The ratio is independent of the alcohol used as starting material. Curiously, however, although either starting material gives a mixture of fluorides, some net

memory of the stereochemistry of the alcohol must be retained, since the ratio of fluorides is different from **84** than from **85**. A scheme consistent with these observations is shown (Scheme 36).

Scheme 36.



Either alcohol gives rise to a mixture of isomers, as is expected with DAST reactions, which proceed through a partially carbocationic intermediate. However, the observation that a different mixture is obtained depending on starting material is most readily explained by assuming that a direct S_N2 displacement mechanism is also taking place competitively. In this case, hexane is the reaction solvent: use of hexane reduces the carbocationic character of the transition state leading to substitution, and hence should favor the S_N2 path. However, the carbocation is stabilised by resonance, and the other resonance form places charge on a tertiary center. The formation of three fluorides is thus readily

explained: the products are the two epimeric 7-fluorosterols, and the (probably α) 5-fluorosterol. The observed fluorine-proton coupling constants are consistent with this assignment. The stability of the resonance-stabilised carbocation is sufficiently high that water or silica treatment regenerates this species. The carbocation is then trapped by water, which attacks it predominantly on the less hindered, α-face, giving **85** as the major product. As is expected with this mechanism, small amounts of a mixture of alkenes are also formed, due to elimination from the carbocation. An alternative mechanism could involve formation of intimate ion pairs in either or both steps: this mechanism could account for the predominant inversion observed.

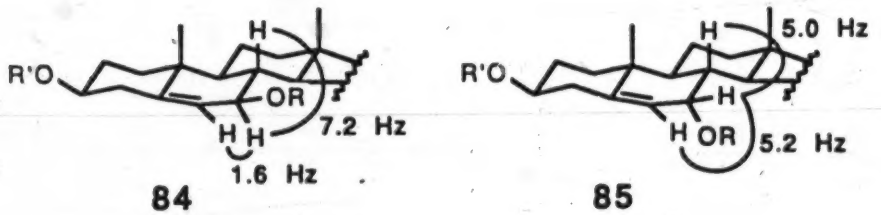
Clearly, the fluorides are too unstable for examination of binding to fungichromin (**1**); however, the introduction of fluorine into the steroid is a novel approach, and may well be worth further investigation.

The DAST reaction does prove useful, since it enables conversion of the major, equatorial alcohol **84** into the minor isomer **85**. If **85** is the desired product, the mixture of alcohols from reduction of **83** may be used directly as starting material. Clean inversion of the stereochemistry of either **84** or **85** is not a simple task. Attempts to convert **84** into the trifluoromethanesulfonate for displacement, using trifluoromethanesulfonic anhydride,[367] result in formation of an intensely blue solution. The same colour has been observed upon treatment of steroidal C-7 alcohols with concentrated sulfuric acid,[368] and is probably due to formation of the carbocation. Stary and Kocovsky[369] report that attempts to invert the stereochemistry of C-7 steroidal alcohols by the Mitsunobu reaction give mixtures of isomers or do not proceed in a satisfactory manner (also compare results described later). This result further emphasises the stability of the C-7 steroidal carbocation, since the Mitsunobu reaction generally proceeds stereospecifically.[370]

Perhaps due to facile inversion of stereochemistry at C-7, conflicting reports on the physical and spectroscopic characteristics of C-7 substituted sterols exist.[368, 371] Therefore, stereochemical assignment of alcohols **84** and **85** is crucial. This assignment is

best achieved by high-field NMR, which was not available at the time of earlier work. For the minor isomer from the reduction (**85**), the proton NMR signal for the olefinic proton (δ 5.55) is a doublet of doublets, J = 5.2 and ca. 1 Hz. In the major isomer (**84**), the olefinic proton (δ 5.24) appears as a doublet of doublets, J = ca. 1.6 and 1.6 Hz. The larger coupling for the minor isomer suggests a dihedral angle close to 0°, while the small coupling in the major isomer suggests a dihedral angle close to 90°. Careful examination of molecular models, important here because the double bond makes the ring adopt a pseudo-chair conformation, supports the proposed structures. Examination of the coupling constants of the C-7 protons in each isomer provides further support. Alcohol **84** shows a broad doublet, J = 7.2 Hz, while **85** shows a broad doublet of doublets, J = 5.0 and 5.0 Hz. The larger coupling in **84** is due to coupling to the proton on C-8, with a dihedral angle slightly below 180° (Figure 18).

Figure 18. Proton NMR coupling constants for assignment of stereochemistry of alcohols **84** and **85**.

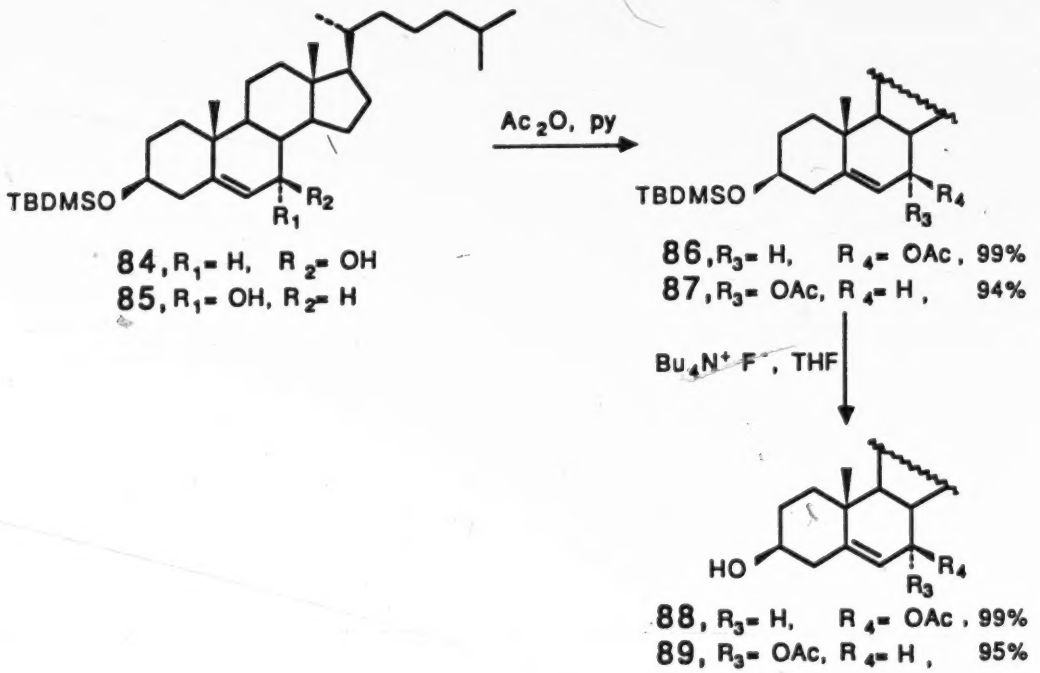


Treatment of **84** with acetic anhydride and pyridine[249] gives acetate **86** cleanly, while **85** affords **87** (Scheme 37). Deprotection of **86** with tetra-n-butylammonium fluoride[362] then gives the desired 7β-acetoxycholesterol (**88**), while similar treatment of **87** affords 7α-acetoxycholesterol (**89**).

Binding studies with **88** and **89** show that the equatorial acetate **88** does not bind fungichromin (**1**); however, axial acetate **89** binds with apparent binding constant K_{app} = (2.22 ± 0.15) X 10^3 M^{-1} and cooperativity n = 2.0 ± 0.1, close to the values for

cholesterol. This result is unexpected, since the widely-held belief that binding is optimal for sterols possessing a flat B-ring would suggest that **89**, with a large acetoxy group lying below the plane of the sterol, and on the less hindered, α-face of the molecule, should bind less effectively. This phenomenon merits further study.

Scheme 37.



84, R_1 = H, R_2 = OH
85, R_1 = OH, R_2 = H

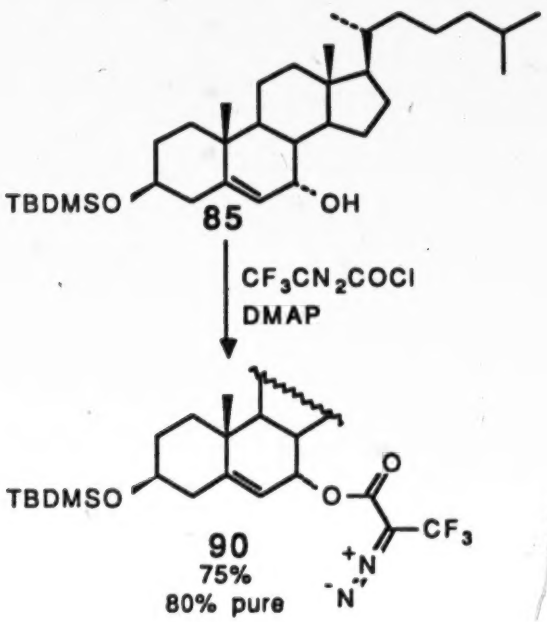
86, R_3 = H, R_4 = OAc, 99%
87, R_3 = OAc, R_4 = H, 94%

88, R_3 = H, R_4 = OAc, 99%
89, R_3 = OAc, R_4 = H, 95%

The model studies of acetates **88** and **89** show that the 7α-isomer is required for photoaffinity labelling experiments. Condensation of alcohol **85** with acid chloride **75** proceeds more efficiently with catalysis by N,N-dimethylaminopyridine, than with prior deprotonation with n-butyllithium (Scheme 38). However, the product **90** is unstable to silica gel chromatography, decomposing back to a mixture of alcohols **84** and **85**. Again, this decomposition is probably due to facile formation of the C-7 carbocation. Rapid flash chromatography[372] allows purification to approximately 80% purity. The material was not purified further, due to problems associated with deprotection.

Initially, the photoaffinity-labelled ester of cholesterol **72** was used to test the stability of the trifluorodiazopropionyl group to possible conditions for deprotection of the

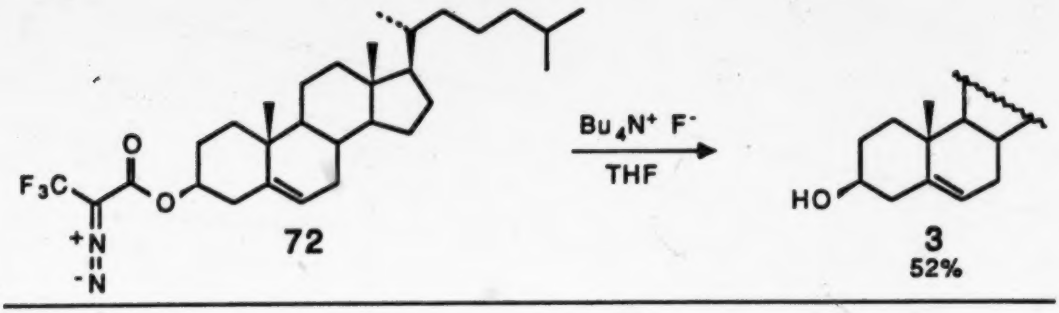
Scheme 38.



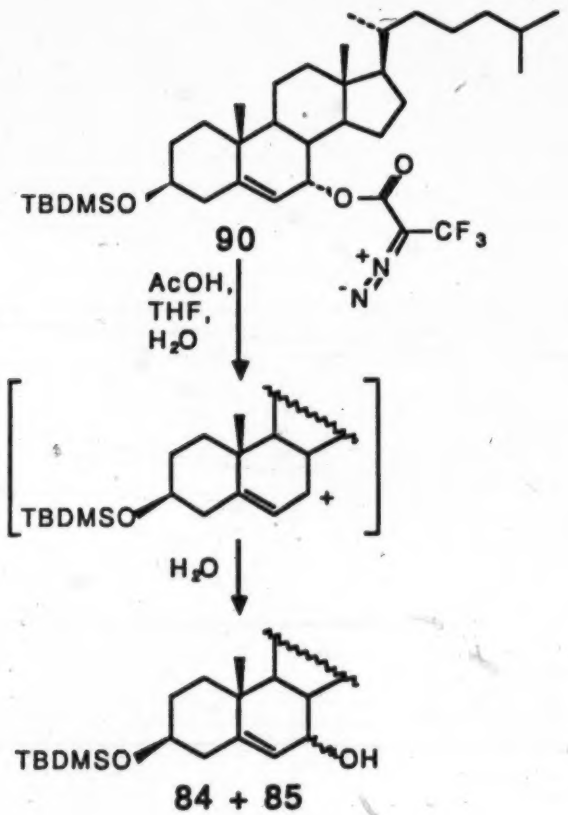
tert-butyldimethylsilyl group of **90**. Treatment of **72** with tetra-n-butylammonium fluoride in THF[362] leads to rapid decomposition. The product shows many signals in the fluorine-19 NMR spectrum, and the fluorine containing products are not readily isolable. However, cholesterol (**3**) is obtained in 52% yield (Scheme 39). A similar result is obtained when **90** is treated in the same manner: little deprotection occurs while the photoaffinity label moiety is completely decomposed. Another method for deprotection of *tert*-butyldimethylsilyl ethers uses aqueous acetic acid and THF at 50 °C.[362] Ester **72** is stable under these conditions, as is expected from the conversion of **74** to **73**, while *tert*-butyldimethylsilyl ether **83** is partially deprotected to **82**. However, **90** decomposes rapidly under the same conditions to form a mixture of alcohols **84** and **85**. This mixture probably results from protonation of the ester, which facilitates elimination to the C-7 carbocation which is then trapped by water (Scheme 40).

Compounds **72** and **86** were used as models in exploring the possible desilylation of **90** by the method of Kendall *et al.*[373] Reaction of **86** with sodium fluoride-

Scheme 39.



Scheme 40.



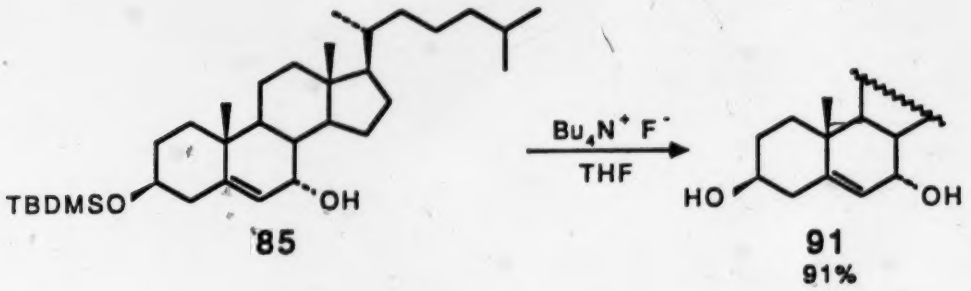
hydrofluoric acid buffer at pH 5 in THF gives only very slow deprotection, while **72** under the same conditions undergoes a faster decomposition. Treatment of model compound **84** with cation exchange resin in methanol and THF using the method of Corey *et al*[374] leads to extensive decomposition to a complex mixture of steroids, including a variety of elimination products. Similar products are observed when **84** reacts with potassium fluoride and 18-crown-6 in acetonitrile.[375] Lithium tetrafluoborate,[376] prepared from lithium hydroxide and fluoboric acid, in dichloromethane and acetonitrile leads to partial deprotection of **90**; however, complete decomposition of the diazo-ester functionality occurs over the same time period. The reaction proceeds by slow decomposition of tetrafluoborate to fluoride, which then effects desilylation. Probably, the fluoride attacks the diazo-ester moiety, as described above in the case of tetra-n-butylammonium fluoride. Clearly, deprotection of the *tert*-butyldimethylsilyl group in the presence of the photoaffinity label is not promising: a more readily cleaved protecting group at C-3 is desirable. Base-labile protecting groups are precluded due to the sensitivity of the diazo-ester to these conditions, while acid-labile protecting groups lead to elimination to the C-7 carbocation.

An exceedingly labile protecting group is the trifluoroacetate ester. Literature precedent[377] suggests that selective trifluoroacetylation at the C-3 hydroxyl group of steroid diols is possible, because C-3 is a relatively unhindered center. Deprotection of 7α-alcohol **85** with tetra-n-butylammonium fluoride in THF gives 7α-hydroxycholesterol (**91**) (Scheme 41). However, reaction of **91** with 1 equivalent of trifluoroacetic anhydride in pyridine gives an inseparable and chromatographically unstable mixture of equal amounts (by NMR) of 3β- and 7α-monotrifluoroacetates, probably along with the di-trifluoroacetate. Thus, in this case, reaction is non-regioselective, at least at room temperature.

To test whether the trifluoroacetate group might be removed selectively in the presence of the trifluorodiazo-ester, the mixture of trifluoroacetates was solvolysed in

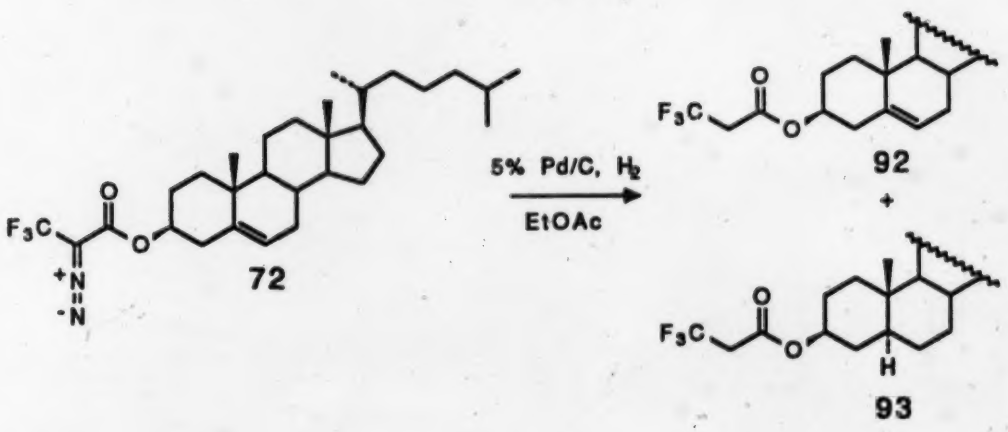
aqueous THF containing phosphate buffer at pH 7, conditions reported to give instant deprotection.[378] However, after 16 hr, some residual sterol trifluoroacetate is still present. Under the same conditions, the model 7α-photoaffinity labelled steroid **90** decomposes more rapidly: none remains by TLC within 90 minutes. Clearly, trifluoroacetate is not a promising protecting group.

Scheme 41.



Catalytic hydrogenation of **72** under conditions that cleave benzyl ethers, for example, leads to decomposition. The major product is an inseparable mixture containing the 3,3,3-tifluoropropionate ester of cholesterol (**92**) and some dihydro compound **93** (Scheme 42). This result indicates that use of a protecting group that is removed under these conditions is not promising.

Scheme 42.



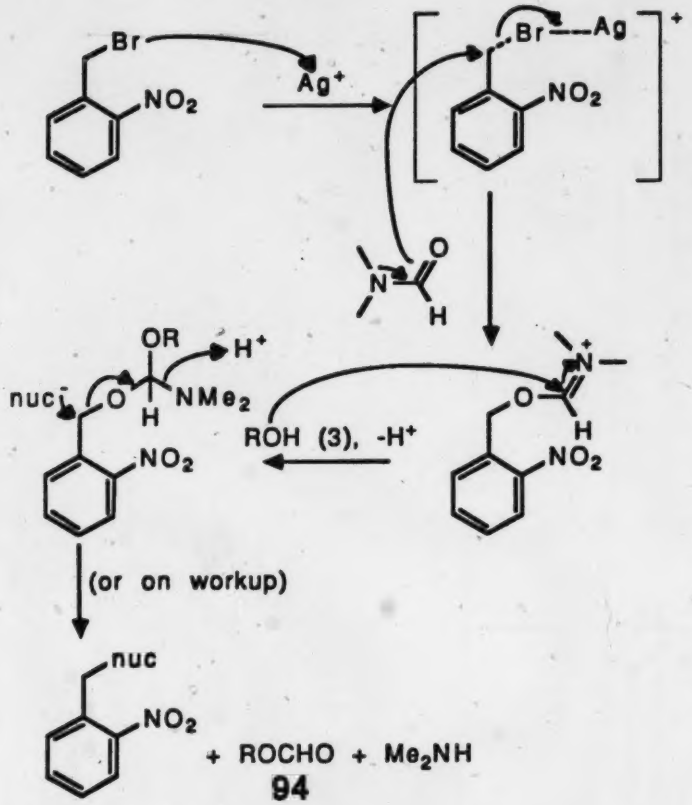
One attractive potential protecting group is the *ortho* -nitrobenzyl ether.[379, 380] This group is readily removed upon photolysis at wavelengths above 300 nm.[381] Since the photoaffinity label moiety absorbs at 238 nm, the simple expedient of photolysis in pyrex apparatus might allow selective photolysis of the protecting group.

However, all attempts to prepare O-(*ortho* -nitrobenzyl)-cholesterol, as a model study, were unsuccessful. Treatment of cholesterol (**3**) with *ortho* -nitrobenzyl bromide and N,N-dimethylaminopyridine fails to give any reaction. Prior deprotonation of **3** with n-butyllithium, or tetramethylammonium hydroxide, followed by addition of *ortho* -nitrobenzyl bromide, also results only in recovered **3**. Although the ring nitro group is expected to destabilise the benzylic carbocation, an S_N1 reaction might afford the desired product. However, treatment of **3** and *ortho* -nitrobenzyl bromide with silver tetrafluoborate in THF gives a complex mixture of products, which does not appear to contain any of the desired material as shown by NMR, TLC or partial purification. In contrast, a similar reaction in DMF gives a new product by TLC, which isolation and spectral analysis shows to be cholesteryl formate (**94**). A possible mechanism (Scheme 43) involves attack by DMF on the cation generated by silver-assisted removal of bromide from *ortho* -nitrobenzyl bromide. Attack by **3** then generates a tetrahedral intermediate which collapses to the formate. Interestingly, no literature precedent appears to exist for *ortho* -nitrobenzyl ethers of secondary alcohols. Preparation of these potentially useful compounds merits further study. Clearly, this protecting group does not appear promising in this case.

The difficulties associated with the deprotection of C-3 protected sterols with a labile substituent at C-7 suggest an approach to the target molecule by acylation with the photoaffinity label as the final step. However, direct selective acylation of diol **91** with acid chloride **75** is unpromising: the facile formation of the photoaffinity labelled ester of cholesterol **72**, compared to the other slower acylations to give **74** and **90**, indicates that reaction will probably proceed predominantly at the C-3 alcohol. Furthermore, the

instability of the C-7 photoaffinity labelled steroid **90** to chromatography indicates that a clean reaction is desired, to allow rapid chromatographic isolation of the desired product. Therefore, an alternative approach involving acylation of a C-7 amine[342] or thiol[382] appears attractive. The enhanced nucleophilicity of these groups over that of the C-7 alcohol might lead to selective acylation at this position.

Scheme 43.



One route to these synthetic targets might be through Mitsunobu reaction on 7β-alcohol **84** with thiol or amine nucleophiles to give the 7α-substituted product.[383] However, treatment of **84** with triphenylphosphine and diethylazodicarboxylate under the usual,[383] or modified,[384, 385] Mitsunobu conditions, followed by treatment with either lithium thiolate or thiolacetic acid, fails to give the desired 7α-substituted product. This

result is in agreement with a literature report describing inability to perform Mitsunobu reactions on C-7 hydroxylated steroids possessing a 5-6 double bond.[369] Alternative routes to the 7α-amino substituted cholesterols are currently under investigation.

Thus, photoaffinity labelling of fungichromin (1) by sterols has not yet been demonstrated. The approach of placing a photoaffinity label as a substituent on the sterol may be fundamentally problematic. The product sterol may not bind if the label is close to the polyene in the complex, due to unfavourable steric effects. However, when these effects are absent, the label moiety may be too far removed from the polyene for the carbene intermediate to attack. This possibility makes the side-chain derivative **73** particularly attractive for other studies of sterol photo-affinity labelling.

The results do, however, suggest a promising approach. Placement of axial or equatorial substituents at different locations on the sterol, followed by examination of the effect on binding, may allow "mapping" of the steric requirements for binding, and hence of the polyene "surface" in the complex.

An investigation into the effects of introducing substituents into the sterol in the theoretical model of Fraga and coworkers would be interesting. The resulting interaction energies relative to that of cholesterol could be compared with experimental relative binding constants.

Conclusions

Sterols have been prepared synthetically by novel or literature procedures to determine the requirement for double bonds in the sterol 'B'-ring and side-chain for optimal binding to polyene antibiotics. Notably, an efficient method for conversion of sterol-5-enes into 5,7-dienes has been developed. A technique for ultraviolet spectroscopy of sterol-polyene mixtures has been developed that gives readily analysed results, and is a rapid method of determining binding constants. However, very little differentiation between the prepared sterols was found in this assay, although some sterol analogues have been shown

not to bind to fungichromin (1). A photoaffinity labelled sterol analogue, with the label in the side-chain, has been prepared, and binds to 1. However, no novel product was detected in the labelling experiment. Both 7α- and 7β-acetoxycholesterol were prepared: the 7α-compound binds well to 1, in spite of the axial substituent. The widely-held belief that a planar B-ring is necessary for efficient binding is not consistent with this result. Further studies should allow determination of the sterol structural requirements for efficient binding to 1.

NMR is a promising technique for probing the structure of polyene-sterol complexes. Full assignment of the proton and carbon NMR spectra of polyenes may be accomplished by a combination of 2-dimensional NMR and biosynthetic experiments. Such an approach may be of use in the structural elucidation of polyene antibiotics, many of which do not have fully known structures. The novel technique of using a mixture of labelled precursors which are relatively efficiently incorporated into, for example, acetate units, may permit rapid determination of the full carbon connectivity pattern of polyketides.

The biosynthetic studies on 1, which are the first on polyenes using stable isotopes, show that the polyene macrolide ring has a polyketide biogenesis. An interesting feature in fungichromin (1) is the unprecedented intact incorporation of octanoate as a unit into a polyketide metabolite. The octanoate derives in turn from degradation of oleate.

Experimental

General

All reactions requiring non-aqueous conditions were performed in oven-dried glassware under a positive pressure of argon. All solvents were distilled. All reagents were recrystallised, or reagent grade or better. Commercially available labelled precursors were purchased from Cambridge Isotope Laboratories (Woburn, MA). Water was from a Millipore purification system. All organic layers obtained from extractions were dried over anhydrous Na_2SO_4. The term *in vacuo* refers to the removal of solvent on a rotary evaporator followed by evacuation to constant sample weight (< 0.05 mm Hg). All reactions were followed by thin layer chromatography (TLC) using either UV fluorescence, iodine staining, or dodecamolybdophosphoric acid for visualisation. Commercial TLC plates were Merck 60F-254 (silica) or Merck RP-8 F-254S (reverse phase). Silica gel for column chromatography was Merck type 60, 70 - 230 mesh. Flash chromatography was performed using the method of Still *et al* [372] on Merck type 60 silica gel, 230-420 mesh. Medium pressure liquid chromatography (MPLC) was performed using either Merck type 60H silica gel (normal phase) or a Merck Lobar™ RP-8 column, size B (reverse phase).

High pressure liquid chromatography (HPLC) was performed using a Hewlett Packard 1082B instrument fitted with a Whatman Partisil™ M9 10/25 column and UV detector set at 254 nm. Gas chromatography (GC) was performed on a Hewlett Packard 5890A gas chromatograph fitted with either a 3% OV-101 on WHP-100-120 6 ' X 1/4 ", or a 80/100 WAW DMCS B8 L11 20 " 10% UCW 982 carbowax column with helium as the carrier gas. Compounds were detected using a flame ionization detector for the former column, or a thermal conductivity detector for the latter.

Temperatures for kugelrohr distillation are those of the air bath surrounding the distillation flask, and do not necessarily represent true boiling points (bp). Melting points

(mp) were determined on either a Thomas Hoover or Buchi apparatus using open capillary tubes and are uncorrected, unless otherwise specified. Nuclear magnetic resonance (NMR) spectra were recorded on Brucker WP-80, WH-200, AM-300, WM-360, WH-400 OR AM-500[193] instruments in the specified deuterated solvent with tetramethylsilane (TMS) as internal standard for ^{1}H spectra, and solvent as internal standard for ^{13}C spectra. All ^{19}F spectra were recorded using $CFCl_3$ as external standard at a chemical shift of 0 ppm. ^{31}P spectra were recorded at 80 MHz using 85% H_3PO_4 as external standard at a chemical shift of 0 ppm. Infrared (IR) spectra were determined on a Nicolet 7199 FT-IR spectrometer. Mass spectra (MS) were recorded at an ionizing voltage of 70 eV on an AEI MS-50 instrument for high resolution electron impact (EI) ionization and on an MS-12 instrument for low resolution EI and for ammonia and isobutane chemical ionization (CI). Fast-atom bombardment mass spectra (FAB-MS) were recorded on an MS-9 instrument. Optical rotations were measured on Perkin-Elmer 241 or 141 polarimeters with a microcell (100 mm, 1 mL). Ultraviolet (UV) spectra were recorded on a Cary 210 or a Pye Unicam SP1700 instrument.

NMR methods

Isotopic incorporations into fungichromin (1) were determined by comparison of the heights of peaks, due to labelled and unlabelled sites, in the ^{1}H-decoupled ^{13}C NMR spectrum, accumulated with a relaxation delay of 1.2 s. Unlabelled 1 gave peaks of uniform height under these conditions. The ^{18}O isotope shifts were determined in the usual fashion[107] by accumulation of the ^{1}H-decoupled ^{13}C NMR spectrum over a narrow window; the fid was then zero-filled once to give a resolution of ca. 0.1 Hz/pt.. The ^{2}H NMR spectra were accumulated at 61.42 MHz using a ^{19}F lock of C_6F_6 and ^{1}H broadband decoupling.

The 2D-INADEQUATE[195] spectra were recorded at 300 MHz. For the polyene region of 1, 64 experiments were performed, accumulating 1152 scans per experiment in a

1K data block over a 625 Hz sweep width centred at δ 132.2. The relaxation delay was 1 s. The value of $J_{(^{13}C-^{13}C)}$ selected was 56 Hz. The data was zero-filled to 512 words in F1, and subjected to Fourier transformation using Gaussian data manipulation in the F1 dim- -ion. The spectrum was then symmetrized for improved appearance. For the polyol and methylene region of 1, 128 experiments were performed, accumulating 1408 scans per experiment in a 2K data block over a 3290 Hz sweep width centred at δ 54.60. The relaxation delay was 1 s. The value of $J_{(^{13}C-^{13}C)}$ selected was 38 Hz. The data was zero-filled to 1K in F1, and subjected to Fourier transformation using Gaussian data manipulation in F1. Again, the spectrum was symmetrized to improve appearance.

For the 2D ^{1}H-^{13}C heteronuclear shift correlation[194] of the polyene and polyol region of 1, 317 experiments were performed on a WH-500[193] instrument accumulating 400 scans per experiment in a 2K data block over a 10640 Hz sweep width centred at δ 102 (F2). The ^{1}H sweep width was 2050 Hz, centred at δ 5.00 (F1). The relaxation delay was 1 s, and the value of $J_{(^{13}C-^{1}H)}$ selected was 131 Hz. The data was zero-filled to 4K in F2 and to 1K in F1 and subjected to Fourier transformation using Lorentzian data manipulation.

For the 2D ^{1}H-^{13}C heteronuclear shift correlation of the methylene and methyl region of 1, 128 experiments were performed on a WH-400 instrument, accumulating 352 scans per experiment in a 2K data block over a 5500 Hz sweep width centred at δ 41.5 (F2). The ^{1}H sweep width was 900 Hz centred at δ 1.86 (F1). The relaxation delay was 1 s, and $J_{(^{13}C-^{1}H)}$ was selected at 130 Hz. The data was zero-filled to 4K in F2 and to 512 words in F1, and was subjected to Fourier transformation using Lorentzian data manipulation in F1.

For the ^{1}H-^{1}H COSY[192] spectrum of the polyene region of 1, 410 experiments were performed on a WH-500[193] instrument, accumulating 48 scans per experiment in a 2K data block over a 500 Hz sweep width centred at δ 6.3. The relaxation delay was 1 s. The data was zero-filled to 1K in F1 and subjected to Fourier transformation using

Gaussian line shaping in F1. The spectrum was symmetrized to improve appearance. In all cases, symmetrization did not affect the overall results obtained.

General procedure for determination of binding constants of sterols to fungichromin (1)

Fungichromin (1) (ca. 2 mg) was accurately weighed, and dissolved in DMSO (3 drops). THF (1.00 mL) was added, and the solution made up to 10.0 mL with degassed H_2O. The solution was stored under Ar, and used immediately.

Sterol (85 - 100 mg) was accurately weighed, and made up to 1.00 mL in THF. Microlitre aliquots (typically to give total additions of 0, 2.5, 5.0, 10.0, 20, 50, 100 and 200 μL) were added successively to 5 mL of the fungichromin (1) solution. After each addition, the mixture was vigorously shaken, and the UV spectrum recorded in 0.1 mm cells over the range 450-220 nm. For all sterols not containing a diene functionality in the B ring, the reference cell contained THF 10%/H_2O 90%. For dienes, improved background cancellation was obtained by adding corresponding microlitre aliquots of sterol to THF 10%/ H_2O 90% (5 mL), and using the resulting suspension in the reference cell. For sterols not containing the diene system, this procedure did not alter the results. In cases where the UV absorption was falling slowly with time, spectra were accumulated until constant values were obtained. Each binding experiment was accompanied by a simultaneous experiment with cholesterol, as a control. In cases where binding did not take place, stock cholesterol solution (50 μL) was added upon completion of the experiment to check for activity. All experiments were performed at 23.0 °C.

The total concentration of sterol present, $[S]_0$, and the change in absorbance at 359 nm, $\Delta A_{359}/d$, where d is the cell path length, were computed, using the formula $\Delta A_{359}/d = (A_0 - A_{obs})/d$, where A_0 is the absorbance when $[S]_0 = 0$, and A_{obs} is the observed absorbance. A plot of $\Delta A_{359}/d$ against $\log[S]_0$ was prepared, from which the maximum change in absorbance, ΔA_{max}, was estimated. The mole fraction of sterol bound, $\hat{y}$, was

then evaluated: $\hat{y} = (\Delta A_{359}/d)/(\Delta A_{max})$. $\hat{y}$ was then plotted against log $[S]_0$; when $\hat{y}$ = 0.5, log $[S]_0$ = log K_{app}, the apparent equilibrium constant of binding. Hill plots were prepared by plotting log $[\hat{y}/(1-\hat{y})]$ against log $[S_0]$.

General procedure for photolysis of photoaffinity labelled sterol - fungichromin mixtures

Fungichromin (**1**) (7.5 mg, 11 µmol) was dissolved in DMSO (7 drops) and THF (1 mL). The photoaffinity label (12 µmol) was added, and the whole made up to 10 mL with degassed H_2O. A 5 mL portion of the degassed suspension was photolysed for ca. 4 h under a slow stream of Ar in a quartz tube using light from a Hanovia medium pressure lamp that had been passed through a diffraction filter (spectral characteristics: λ_{min} 227 nm (A=0.7), A>2 below 202 nm and above 265 nm); the other 5 mL of solution was kept in the dark under Ar for the same length of time. In both cases, aliquots were removed at various times and examined by UV spectroscopy to monitor decomposition or photolysis of the photoaffinity label and stability of fungichromin. Both samples were then separately diluted (EtOH, 10 mL) and concentrated *in vacuo*.

The experiment was then repeated with the omission of fungichromin.The resulting 4 samples were compared by TLC, ^1H and ^{19}F NMR (400 and 376 MHz, $CDCl_3$ 50%/ CD_3OD 50%), and UV (DMSO 10%/ THF 90%).

General procedure for growth of *Streptomyces cellulosae*, and isolation of fungichromin (1).

Freeze-dried specimens of *Streptomyces cellulosae* (ATCC 12625) were soaked in H_2O (1 mL) for 5 min, and transferred to 10 slants, prepared from bacto-yeast malt extract agar (19 g) and H_2O (500 mL), which had been sterilised at 121 °C for 20 min. The slants were incubated at 25 °C for 7 days. The resulting mycelium was suspended in H_2O (2 mL), and the suspension added to 2 Erlenmeyer flasks (500 mL), each containing liquid

media (100 mL) prepared from bactopeptone (5 g), DIFCO yeast extract (2.5 g), NaCl (4 g), glucose (10 g) and Span 85 (Sigma, 10 mL) made up to 1 L with H_2O, buffered to pH 7.0 with $NaHCO_3$, then autoclaved at 121 °C for 20 min. The preculture flasks were incubated in a fermenter at 26 °C and 165 rpm in the dark for 48 h. A 2 mL portion of the resulting suspension was then transferred to each of 10 flasks containing medium prepared as above (100 mL/flask); the flasks were then incubated under the same conditions. After 3 - 4 days, the contents became yellow, and isotopically labelled precursors (for labelled acetates, propionates, hexanoate, and octanoates: 500 mg in 10 mL H_2O; for **16a** and **17a**, and experiments with mixtures of labelled diethyl malonates: 500 mg in 5 mL EtOH and 5 mL H_2O) were added aseptically in 4 portions at 24 h intervals. At 24 h after the last feeding, the mycelium (ca. 25 g fresh weight) was collected by vacuum filtration. The filtrate was extracted (hexane 66%/benzene 34%, 2 X 500 mL, then EtOAc, 2 X 500 mL). The mycelium was gently boiled in hexane 66%/benzene 34% (500 mL, 30 min). The cooled mixture was filtered, and the filter cake extracted with boiling EtOAc (500 mL, 10 min). The combined EtOAc extracts were concentrated *in vacuo* to afford ca. 1.3 g of yellow solid, which was taken up in MeOH, and filtered. The filtrate was concentrated *in vacuo* . Column chromatography of the residue on Sephadex LH-60 (MeOH) afforded UV-active fractions which were concentrated *in vacuo* . The residue was taken up in MeOH (5 mL), H_2O (2.7 mL) was added, and the thick precipitate removed on a centrifuge. Medium pressure liquid chromatography (reverse phase, MeOH 65%/H_2O 35%, 1 mL/min, 6 mL fractions) of the supernatant (5 mL) afforded 20-40 mg of **1** from fractions 35-40 after azeotropic removal of solvent *in vacuo* with EtOH: R_f (SiO_2, $CHCl_3$ 22%/MeOH 22%/EtOAc 45%/H_2O 11%, lower phase) **1**, 0.31; **2**, 0.38. R_f (RP-8, MeOH 65%/H_2O 35%) **1**, 0.35; **2**, 0.27; ^1H and ^{13}C NMR spectral data is given in Table 1; FAB-MS (glycerol-sulfolane matrix) m/e 693 (MNa^+), 670 (M^+).

Modified procedure for fermentation in D_2O

The procedure outlined above was followed, except that H_2O (1 l) in the culture flasks was replaced by D_2O 20%/H_2O 80%.

Modified procedure for fermentation under $^{18}O_2$ atmosphere

The procedure outlined above was followed, except that fermentation flasks were grown in a closed atmosphere in which consumed oxygen was replenished with $^{18}O_2$ (50 atom % ^{18}O), as has been previously described[106, 185].

Modified procedure for fermentation with [U-^{14}C]glucose or [U-^{13}C]glucose

The procedure outlined above was followed, with the following exceptions: for [U-^{14}C]glucose, 2.55 μCi of [U-^{14}C]glucose was added to each of 9 fermentation flasks just prior to innoculation with the preculture. For [U-^{13}C]glucose, 1 g of [U-^{13}C]glucose (Cambridge Isotopes, 99 atom % ^{13}C) totally replaced the glucose in one culture flask; fungichromin (**1**) was isolated with the aid of material from 5 unlabelled "carrier" flasks.

Modified procedure for fermentation with ethyl oleate or ethyl [CD_3]oleate (18a) and ethyl [U-^{14}C]oleate (18b)

The procedure above was followed, except that Span 85 was omitted from the preculture medium, and replaced in the culture medium by ethyl oleate (9 g/litre of medium), **18a** (1 g/litre) and **18b** (22.1 μCi/litre).

Hexyl *p*-toluenesulfonate (13a)[223] and [1-^{13}C]hexyl *p*-toluenesulfonate (13b)

The procedure of H.C. Brown[222] was followed. Thus, hexanoic acid or [1-^{13}C]hexanoic acid (750 mg, 6.40 mmol) was dissolved in Et_2O (15 mL), and $NaBH_4$

(620 mg, 16.4 mmol) was added. The mixture was stirred, and $BF_3.Et_2O$ (48% BF_3, 2 mL, 12.6 mmol) added dropwise over 30 min. The mixture was stirred, and monitored by GLC (3% OV-101 column) until starting acid was no longer present (2 days). H_2O (50 mL) was added, and the mixture was extracted (CH_2Cl_2, 2 X 50 mL). The extracts were washed (5% Na_2CO_3, 100 mL) and dried. Pyridine (6 mL) and *p*-toluenesulfonyl chloride (1.5 g, 7.62 mmol) were added, and the mixture was stirred, and monitored by GLC (as above). After 64 h, the mixture was extracted (saturated $CuSO_4$ (2 X 100 mL), 10% $NaHCO_3$ (100 mL), and H_2O (100 mL)). The organic phases were dried and concentrated *in vacuo* to give 1.44 g (87% based on hexanoic acid) of **13** as a pale yellow semi-solid. For **13a**: IR ($CHCl_3$ cast) 3060, 3030 (Ar-H), 2958, 2931, 2860 (C-H), 1600, 1468, 1360 (str), 1189 (str), 1177 (str), 1100, 929 (str), 816, 665 (str); ^1H NMR (200 MHz, $CDCl_3$) δ 7.79, 7.36 (AB, 8.0 Hz, 4H, ArH), 4.03 (t, 6.4 Hz, 2H, CH_2OTs), 2.45 (s, 3H, $ArCH_3$), 1.64 (pent, 2H, CH_2CH_2OTs), 1.24 (br m, 6H, 3X CH_2), 0.85 (t, 6.4 Hz, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 144.43 and 133.01 (wk, ArC), 129.56 and 127.55 (4C, ArCH), 70.46 (CH_2OTs), 30.78, 28.50, 24.72, 22.10, 21.27, 13.59 (CH_3); exact mass: 256.1133 (256.1133 calcd for $C_{13}H_{20}SO_3$). Anal. calcd for $C_{13}H_{20}SO_3$: C, 60.91; H, 7.86; S, 12.51. Found: C, 60.66; H, 7.99; S, 12.24. For **13b**, spectra were as **13a**, except for: ^1H NMR, δ 4.03 (d, $^1J_{(^{13}C-^1H)}$= 130 Hz); ^{13}C NMR: δ 70.46 (enriched), 30.78 (d, $^2J_{(^{13}C-^{13}C)}$= 4 Hz), 28.50 (d, $^1J_{(^{13}C-^{13}C)}$= 38 Hz).

Diethyl hexylmalonate (14a)[225] and diethyl [1-^{13}C]hexylmalonate (14b)

A modification of the method of Marshall *et al*[224] was used. DMF (5 mL) was added to NaH (50% oil dispersion, 132 mg, 2.74 mmol) which had been washed with THF. Diethyl malonate (327 mg, 2.09 mmol) was added, and the mixture was stirred until effervescence ceased. A solution of **13** (446 mg, 1.74 mmol) in DMF (5 mL) was added, and the mixture was stirred at 70 °C for 16 h. H_2O (100 mL) was added, the mixture was acidified with concentrated HCl, and extracted ($CHCl_3$, 2 X 100 mL). The extracts were

dried and concentrated *in vacuo*. Column chromatography ($CHCl_3$) afforded 226 mg (54%) of **14** as a clear oil. For **14a**: IR ($CHCl_3$ cast) 2959, 2931, 2855 (C-H), 1753 and 1736 (C=O), 1462, 1365, 1035 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.19 (q, 7.2 Hz, 4H, 2 X $C\underline{H}_2O$), 3.31 (t, 7.6 Hz, 1H, $C\underline{H}(CO_2Et)_2$), 1.88 (m, 2H, $C\underline{H}_2CH(CO_2Et)_2$), 1.22-1.38 (m, 8H, 4 X $C\underline{H}_2$), 1.27 (t, 7.2 Hz, 6H, 2 X $C\underline{H}_3CH_2O$), 0.88 (t, 6.8 Hz, 3H, $C\underline{H}_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.29 (2 X $\underline{C}$=O), 60.88 ($\underline{C}H(CO_2Et)_2$), 51.83 (2 X $\underline{C}H_2O$), 31.28, 28.63, 28.51, 27.02, 22.56, 13.80 (2 X $\underline{C}H_3CH_2O$), 13.69 ($\underline{C}H_3$); exact mass: 244.1676 (244.1675 calcd for $C_{13}H_{24}O_4$). Anal. calcd for $C_{13}H_{24}O_4$: C, 63.91; H, 9.90. Found: C, 63.58; H, 9.85. For **14b**, spectra were as **14a**, except for: ^1H NMR δ 3.31 (d, $^2J_{(^{13}C\text{-}^1H)}$= 4.6 Hz), 1.88 (d, $^1J_{(^{13}C\text{-}^1H)}$= 130 Hz); ^{13}C NMR δ 28.51 (enhanced).

Sodium octanoate (10c)[225] and sodium [3-^{13}C]octanoate (10b)

A modification of the method of Anker[225] was used. Aqueous 10% NaOH (10 mL) was added to a solution of **14** (175 mg, 729 μmol) in dioxan (5 mL). The mixture was stirred for 24 h, then acidified to pH 1 with concentrated HCl, and heated at reflux for 36 h. The mixture was cooled and extracted (Et_2O, 3 X 20 mL). The extracts were dried and concentrated *in vacuo*. The residue was adjusted to pH 8.5 with NaOH, and EtOH (30 mL) was added. Organic solvents were removed, and the aqueous solution was lyophilised to afford 120 mg (100%) of **10** as a white powder. For **10c**: ^1H NMR (360 MHz, D_2O) δ 2.08 (t, 7.5 Hz, 2H, $C\underline{H}_2CO_2$), 1.44 (m, 2H, $C\underline{H}_2CH_2CO_2$), 1.18 (br m, 8H, 4 X $C\underline{H}_2$), 0.76 (t, 6.3 Hz, 3H, $C\underline{H}_3$); ^{13}C NMR (90 MHz, D_2O) δ 184.04 ($\underline{C}$=O), 38.30, 31.76, 29.53, 28.97, 26.51, 22.59, 13.95 ($\underline{C}H_3$). For **10b**, spectra were as for **10c**, except: ^1H NMR δ 2.08 (d, $^2J_{(^{13}C\text{-}^1H)}$= 3.2 Hz), 1.44 (d, J obscure); ^{13}C NMR δ 26.51 (enhanced).

p -Phenylphenacyl [3-^{13}C]octanoate (15a)

The method of Risley *et al* [226] was used. A mixture of *p* -phenylphenacyl bromide (33.4 mg, 134 μmol), **10b** (22.7 mg, 121 μmol) and anhydrous EtOH (1 mL) was heated at reflux for 16 h. The mixture was cooled, and H_2O (25 mL) was added. The mixture was extracted ($CHCl_3$, 2 X 25 mL), and the extracts were dried and concentrated *in vacuo*. Column chromatography (benzene) afforded 12.5 mg (30%) of pure **15a**; an analytical sample was recrystallised (EtOH/H_2O) to give pure white **15a**: mp 64.5 - 65.5 °C; IR ($CHCl_3$ cast) 3050 (wk, Ar-H), 2950, 2923, 2845 (C-H), 1750 (C=O ester), 1699 (ArC=O), 1171, 760 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.02, 7.73 (AB, 8 Hz, 4H, disubst. ArH), 7.40-7.70 (m, 5H, monosubst. ArH), 5.41 (s, 2H, OCH_2CO), 2.53 (m, 2H, CH_2CO_2), 1.06 (p, 7 Hz, 1H, part of $^{13}CH_2CH_2CO_2$), 1.34 (m, 9H, 4 X CH_2 and part of $^{13}CH_2CH_2CO_2$), 0.92 (t, 7 Hz, 3H, CH_3); exact mass: 339.1915 (339.1915 calcd for $C_{21}H_{26}{}^{13}CO_3$, 5%), 338 not observed (<1% ^{12}C isotopomer).

Sodium [1-^{13}C, $^{18}O_2$]propionate (19)[237, 238]

The synthesis of Cane *et al* [237, 238] was used. Thus, ethyl iodide (3.07 g, 19.7 mmol), potassium [^{13}C]cyanide (Cambridge Isotopes, >99% ^{13}C, 1.25 g, 18.9 mmol), $H_2{}^{18}O$ (90% ^{18}O, 0.82 g, 41 mmol) and anhydrous MeOH (3.9 mL) were heated to reflux at 70 °C for 5 h, then at 80 °C for 40 h. The mixture was distilled, and the distillate treated with $H_2{}^{18}O$ (0.82 g, 41 mmol) and a solution of potassium *t* -butoxide in *t* -butyl alcohol (22.1 mmol in 26 mL). The mixture was heated at reflux for 48 h, then cooled and concentrated *in vacuo*. The residue was taken up in H_2O (10 mL) and treated with Biorad AG^{TM} 50W-X8 ion exchange resin. The mixture was stirred for 10 min and filtered; the filtrate was adjusted to pH 10 with 10 M NaOH, and lyophilised to afford 1.13 g (60%) of **19** as a white powder: ^{1}H NMR (400 MHz, D_2O) δ 2.18 (d, $^2J_{(^{13}C-^1H)}$= 7.4 Hz, q, 8.0 Hz, 2H, CH_2), 1.07 (d, $^3J_{(^{13}C-^1H)}$= 4.8 Hz, t, 8.0 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, D_2O) δ 189.81 ($\underline{C}O_2$, enhanced), 35.26 (d, $^1J_{(^{13}C-^{13}C)}$= 51.5 Hz, $\underline{C}H_2$), 14.63

($\underline{C}H_3$).

_p_ -Phenylphenacyl [1-^{13}C,$^{18}O_2$]propionate (20)[237]

The method of Cane *et al* [237] was used. A mixture of **19** (21.1 mg, 213 µmol), *p* -phenylphenacyl bromide (132 mg, 480 µmol) and 18-crown-6 (16.9 mg) was heated to reflux in benzene (1 mL) and CH_3CN (1 mL) for 150 min. The mixture was concentrated *in vacuo* . Column chromatography (benzene) gave 48.0 mg (83%) of **20**. An analytical sample was prepared by recrystallisation from EtOH/H_2O: mp 101.5 - 102 °C, (lit.[237, 238] mp 103 °C); IR ($CHCl_3$ cast) 2980 (wk, C-H), 1696 (ArC=O), 1670 ($^{13}C=^{18}O$), 1159, 766 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.99 (m, 2H, Ar$\underline{H}$), 7.36-7.73 (m, 7H, Ar$\underline{H}$), 5.38 (d, $^3J_{(^{13}C-^1H)}$= 4.4 Hz, 2H, $C\underline{H}_2O$), 2.54 (d, $^2J_{(^{13}C-^1H)}$= 7.4 Hz, q, 7.4 Hz, 2H, $C\underline{H}_2CO_2$), 1.23 (d, $^3J_{(^{13}C-^1H)}$= 5.8 Hz, t, 7.4 Hz, 3H, $C\underline{H}_3$); ^{13}C NMR (90 MHz, $CDCl_3$) δ 173.12 ($^{13}\underline{C}O_2$, enriched), 146.38, 139.50 and 132.85 (wk, $\underline{Ar}C$), 128.75, 128.15, 128.10, 127.15 and 127.00 ($\underline{Ar}CH$), 64.53 ($\underline{C}H_2O$), 25.07 (d, $^2J_{(^{13}C-^1H)}$= 58 Hz, $\underline{C}H_2{}^{13}CO_2$), 9.54 ($\underline{C}H_3$); MS (EI) For M^+: m/e 268 ($^{12}C^{16}O_2$, 0%), 269 ($^{13}C^{16}O_2$, 1.6%), 270 ($^{12}C^{18}O^{16}O$, 0.6%), 271 ($^{13}C^{18}O^{16}O$, 19.3%), 272 ($^{12}C^{18}O_2$ and $^{13}C_2{}^{18}O^{16}O$, 5.8%), 273 ($^{13}C^{18}O_2$, 61.6%), 274 ($^{13}C_2{}^{18}O_2$, 10.6%), best fit for 96% ^{13}C, 86% ^{18}O/atom.

Sodium [1-^{13}C, $^{18}O_2$]octanoate (10a) and *p* -phenylphenacyl derivative (15b)

Compound **10a** was prepared (Dr. H. Noguchi) by the method used to prepare **19**,[237, 238] from $K^{13}CN$ and heptyl iodide.

Derivative **15b** was prepared (P. Harrison) by the method used to prepare **20**.[237] Thus, **10a** (7.11 mg, 41.5 µmol) afforded 2.45 mg (17%) of **15b**; spectra were as for **15a**, except for: IR 1671 ($^{13}C=^{18}O$); ^1H NMR δ 5.37 (d, $^3J_{(^{13}C-^1H)}$= 4.5 Hz, 2H, $OC\underline{H}_2CO$), 2.50 (d, $^2J_{(^{13}C-^1H)}$= 7.3 Hz, t, 7.3 Hz, 2H, $C\underline{H}_2CO_2$), 1.72 (m, 2H,

$CH_2CH_2CO_2$); ^{13}C NMR δ 174.01 ($^{13}C{=}^{16}O$), 173.97 ($^{13}C^{18}O_2$), 173.98 ($^{13}C{=}^{18}O(^{16}O)$); MS (EI) m/e 338 ($^{12}C^{16}O_2$, 0%), 339 ($^{13}C^{16}O_2$, 1.9%), 340 ($^{12}C^{16}O^{18}O$, 0%), 341 ($^{13}C^{16}O^{18}O$, 26.2%), 342 ($^{13}C_2{}^{16}O^{18}O$, $^{12}C^{18}O_2$, 5.6%), 343 ($^{13}C^{18}O_2$, 53.8%), 344 ($^{13}C_2{}^{18}O_2$, 12.5%). Best fit for: ^{13}C, 100%; ^{18}O 71.5 and 91.9%/site.

S-Octanoyl-N-acetylcysteamine (17a) and S-[1-^{13}C]octanoyl-N-acetylcysteamine (17b)

The procedure of Kass and Brock[230] was used. To octanoic acid or [1-^{13}C]octanoic acid (Cambridge Isotopes, 99% ^{13}C) (360 mg, 2.50 mmol) in THF (50 mL) at 0 °C under Ar was added Et_3N (250 mg, 2.5 mmol), then ethyl chloroformate (270 mg, 2.5 mmol). The mixture was stirred at 0 °C for 1h.

N,S-Diacetylcysteamine (a generous gift from Dr. B.J. Rawlings, University of Alberta) (1.21 g, 7.5 mmol) was dissolved in H_2O (40 mL) containing KOH (1.40 g, 25 mmol). Ar was bubbled through the solution, which was stirred for 45 min. The pH was adjusted to 7.8 with concentrated HCl, and the mixture added to the above solution. Bubbling of Ar through the mixture was continued, and the cooling bath was removed. H_2O was added to maintain homogeneity, the pH was adjusted to 8.0 ± 0.1, and this pH was maintained while the mixture was stirred for 1h. The pH was adjusted to 3.0 and THF was removed on a rotary evaporator. The aqueous residue was extracted (Et_2O, 2 X 100 mL) and the organic layers washed (1 M HCl, 200 mL), dried and concentrated *in vacuo*. The residue was crystallised from pentane containing a trace of ethanol to afford 525 mg (86%) of pure **17** as a white solid; for **17a**: mp 56 - 56.5 °C; IR ($CHCl_3$ cast) 3293 (br, N-H), 2953, 2922, 2857 (C-H), 1690 (C=O amide), 1638 (C=O thioester); ^1H NMR (200 MHz, $CDCl_3$) δ 6.06 (br s, 1H, NH), 3.42 (q, 6.2 Hz, 2H, CH_2N), 3.02 (t, 6.2 Hz, 2H, CH_2S), 2.56 (t, 7.3 Hz, 2H, CH_2CO), 1.96 (s, 3H, CH_3CO), 1.65 (p, 7.2 Hz, 2H, CH_2CH_2CO), 1.26 (br m, 8H, 4 X CH_2), 0.87 (t, 6.6 Hz, 3H, CH_3); ^{13}C NMR (50

MHz, $CDCl_3$) δ 199.94 (C=O thioester), 170.51 (C=O amide), 44.16, 39.72, 31.61, 28.90, 28.90, 28.46, 25.68, 23.08, 22.57, 14.01; UV (dioxan) λ_{max} (log ε) 234 (3.576), 237 (3.534) nm, (lit.[230] for analogous adducts: 230 - 233 (3.602) nm); exact mass: 245.1446 (245.1442 calcd for $C_{12}H_{23}NO_2S$). Anal. calcd for $C_{12}H_{23}NO_2S$: C, 57[1].74; H, 9.45; N, 5.71; S, 13.06. Found: C, 57.80; H, 9.20; N, 5.71; S, 13.14. For **17b**, spectra were as for **17a**, except for: ^1H NMR δ 3.02 (d, $^3J_{(^{13}C-^1H)}$=4.6 Hz), 2.56 (d, $^2J_{(^{13}C-^1H)}$= 5.6 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 199.93 (enhanced); exact mass: 246.1478 (246.1473 calcd for $C_{11}H_{23}{}^{13}CO_2NS$), no peak at m/e 245, isotopic purity ≥ 98% ^{13}C.

General procedure for preparation of labelled esters (11, 16 and 18) from the parent carboxylic acids[§]

The method of Boissonnas *et al* [209] was used. Thionyl chloride (1.1 equiv. for octanoic and oleic acids, 2.2 equiv. for malonic acids) was added to EtOH (1 mL/ mmol acid). The carboxylic acid was then added, the mixture stirred for 30 min, solvent was removed, and the residue distilled in a kugelrohr.

For **16**: octanoic acid or [1-^{13}C]octanoic acid (640 mg, 4.44 mmol) afforded 650 mg (85%) of **16**, distilled at 95 - 100 °C at 20 mm Hg. For **16a**: IR ($CHCl_3$ cast) 2955, 2925, 2855 (C-H), 1739 (C=O), 1175, 1165 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 4.03 (q, 7.2 Hz, 2H, CH_2O), 2.20 (t, 7.4 Hz, 2H, CH_2CO_2), 1.52 (p, 7.2 Hz, 2H, $CH_2CH_2CO_2$), 1.20 (br m, 8H, 4 X CH_2), 1.14 (t, 7.2 Hz, 3H, CH_3CH_2O), 0.78 (t, 6.6 Hz, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.53 (C=O), 59.85, 34.20, 31.50, 28.95, 28.76, 24.84, 22.41, 14.04, 13.79; exact mass: 172.1461 (172.1463 calcd for $C_{10}H_{20}O_2$). For **16b**, spectra were as **16a**, except for: ^1H NMR δ 4.03 (d, $^3J_{(^{13}C-^1H)}$=

[§]The procedure for preparation of **18** from oleic acid was done by Dr. K. Arai.

3.0 Hz), 2.20 (d, $^{2}J_{(^{13}C-^{1}H)}$= 7.2 Hz), 1.54 (d, $^{3}J_{(^{13}C-^{1}H)}$= 4.8 Hz); ^{13}C NMR δ 173.53 (enhanced), 34.20 (d, $^{1}J_{(^{13}C-^{13}C)}$= 57 Hz, $\underline{C}H_2{}^{13}CO_2$), 28.95 (d, $^{2}J_{(^{13}C-^{13}C)}$= 8 Hz, $\underline{C}H_2CH_2{}^{13}CO_2$); exact mass: 173.1495 (173.1496 calcd for $C_9H_{20}{}^{13}CO_2$), no peak at m/e 172: isotopic purity ≥ 95% ^{13}C.

For **11**: malonic acid, [1,3-$^{13}C_2$]malonic acid or 100 μCi [2-^{14}C]malonic acid (990 mg, 9.51 mmol) afforded 1.27 g (83%) of **11**, distilled at 95 - 100 °C at 35 mm Hg. For diethyl malonate, ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.18 (q, 8.0 Hz, 4H, 2 X CH_2O), 3.34 (s, 2H, $CH_2(CO_2Et)_2$), 1.25 (t, 8.0 Hz, 6H, 2 X CH_3); ^{13}C NMR (90 MHz, $CDCl_3$) δ 166.48 (2C, 2 X $\underline{C}$=O), 61.36 (2C, 2 X $\underline{C}H_2CH_3$), 41.65 ($\underline{C}H_2(CO_2Et)_2$), 13.98 (2C, 2 X $\underline{C}H_3$). For **11a**, spectra were as for diethyl malonate, except for: ^{1}H NMR δ 4.18 (d, $^{3}J_{(^{13}C-^{1}H)}$= 3.0 Hz), 3.34 (t, $^{2}J_{(^{13}C-^{1}H)}$= 8.0 Hz, $CH_2{}^{13}C_2$, and d, 8.0 Hz, $CH_2{}^{13}C{}^{12}C$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.48 (enhanced), 41.66 (t, $^{1}J_{(^{13}C-^{13}C)}$= 59 Hz, $\underline{C}H_2{}^{13}C_2$).

For **18**: a mixture of [CD_3]oleic acid (500 mg, 1.75 mmol) and [U-^{14}C]oleic acid (23.0 μCi) afforded 505 mg (92%) of **18a** and **18b** (22.1 μCi, 96% radiochemical yield), distilled at 200 - 210 °C at 10 mm Hg.

3-β-Acetoxycholest-5-ene (5)[250]

The method of Staunton and Eisenbraun[249] was modified. A mixture of **3** (2.51 g, 6.50 mmol) and acetic anhydride (4 mL, 42.4 mmol) was heated to reflux for 10 min. The crystals obtained on cooling were collected, washed with cold MeOH, and recrystallised from EtOH to give 2.43 g (87%) of **5** as white needles: mp 113 - 113.5 °C, (lit.[250] mp 115 - 116 °C); $[\alpha]_D{}^{24}$ -42.0° (*c* 2.17, $CHCl_3$), (lit.[250] $[\alpha]_D$ -47.7° (*c* 2, $CHCl_3$)); IR ($CHCl_3$ cast) 2960, 2910, 2870 (C-H), 1730 (C=O), 1255, 1040 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.40 (br d, 4.4 Hz, 1H, $C\underline{H}$=C), 4.62 (m, 1H, $C\underline{H}$OAc), 2.32 (br d, 8 Hz, 2H, 4-CH_2), 2.04 (s, 3H, CH_3CO_2), 1.04-2.10 (m, 26H, $C\underline{H}$, $C\underline{H}_2$), 1.020 (s, 3H, CH_3-19), 0.914 (d, 6.6 Hz, 3H, CH_3-21), 0.864 (d, 6.6 Hz, 6H, CH_3-26, CH_3-27),

0.675 (s, 3H CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.42 (C=O), 139.75 (wk, C=CH), 122.67 (CH=C), 74.01 (CHOAc), 56.79, 56.28, 50.16, 42.39, 39.83, 39.59, 38.20, 37.08, 36.64, 36.27, 35.84, 31.95, 31.95, 28.26, 28.03, 27.84, 24.32, 23.91, 22.82, 22.58, 21.37, 21,10, 19.33, 18.77, 11.89; exact mass: 428.3658 (428.3654 calcd for $C_{29}H_{48}O_2$, 0.1%), 368.3438 (M^+-AcOH, 100%). Anal. calcd for $C_{29}H_{48}O_2$: C, 81.25; H, 11.29. Found: C, 81.29; H, 11.17.

t-Butyl hypochlorite (25)[252]

The method of Mintz and Walling[252] was used. To aqueous sodium hypochlorite (2 L), cooled to 10 °C, was added a mixture of *tert*-butyl alcohol (49 mL, 520 mmol) and acetic acid (51 mL, 890 mmol) in one portion. The mixture was stirred for 3 min, and the yellow organic layer collected, washed (10% Na_2CO_3, 200 mL, then H_2O, 200 mL) and dried over $CaCl_2$ to afford 56 g (100%) of **25** which was used immediately.

N-Phenyltriazoline-3,5-dione (24)[251]

The method of Cookson *et al*[251] was used. Compound **25** (6.20 mL, 54.7 mmol) was added to a solution of N-phenylurazole (8.79 g, 49.6 mmol) in dioxan (250 mL). The bright red solution was stirred for 30 min and concentrated *in vacuo*. The red solid was sublimed (80 °C, 0.1 mm Hg) to afford 6.22 g (72%) of **24** as red cubic crystals: IR (dioxan cast) 1770, 1750 (C=O) cm^{-1}, (lit.[251] 1780, 1760 cm^{-1}); UV (dioxan) λ_{max} (log ε) 246 (3.30), 305 (infl., 2.90), 527 (2.19), (lit.[251] 242-260 (3.45), 300 (3.02), 526 (2.27)).

3β-Acetoxycholesta-5,7-diene N-phenyltriazoline-3,5-dione adduct (26)[386]

Allylic bromination/dehydrobromination was done according to the procedure of Bernstein *et al*.[241] Triazolinedione adduct formation used a modification of the method of Aberhart *et al*.[254] Thus, a mixture of **5** (3.44 g, 8.02 mmol), and N-bromosuccinimide

(1.72 g, 9.67 mmol) in alkene-free hexane (40 mL) was brought to reflux and irradiated under the influence of a heat lamp and a UV spotlight (GE, 275 W) for 40 min. s-Collidine (1.6 mL, 12.1 mmol) was added, and the mixture was cooled. The solid was collected, and washed with hexane (2 X 20 mL). The combined filtrates were concentrated *in vacuo* . A solution of *s* -collidine (0.8 mL, 6.1 mmol) in *p* -xylene (20 mL) was added to the red oily residue, and the mixture was heated to reflux for 15 min. Water (20 mL) was added, and the mixture was extracted with *p* -xylene (2 X 20 mL). The combined organic layers were washed (H_2O, 50 mL), dried and concentrated *in vacuo*. Column chromatography ($CHCl_3$) afforded a mixture of non-polar sterol acetates (3.22 g), which was dissolved in EtOAc (100 mL). The solution was cooled to 0 °C, and titrated with a solution of **24** in EtOAc. 855 mg of **24** was required, equivalent to 61% yield of diene. Excess **24** (50 mg) was added and the mixture stirred at 0 °C for 10 min. Alumina (Woelm grade V, 5 g) was added and the mixture allowed to warm to room temperature, and filtered. The alumina was washed (EtOAc, 2 X 50 mL), and the combined filtrates concentrated *in vacuo* . Column chromatography ($CHCl_3$, then MeOH 2%/$CHCl_3$ 98%) afforded:

Fraction (1), recovered sterol acetates (a mixture).

Fraction (2) 2.51 g (53%) of **26** as a pale yellow foam: mp 86 - 88 °C (phase change), 98 - 99 °C (melt), (lit.[386] mp 133 - 136 °C); $[\alpha]_D^{24}$ -64.1° (*c* 1.9, $CHCl_3$), (lit.[386] $[\alpha]_D^{21}$ -91.4° (*c* 2.26, $CHCl_3$)); IR ($CHCl_3$ cast) 2952, 2930, 2865 (C-H), 1752 (C=O amide), 1735 (C=O ester), 1704 (str, C=O amide), 1400, 1243, 755 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.47, 7.32 (m, 5H, ArH), 6.42, 6.23 (AB, 8.4 Hz, 2H, CH=CH), 5.46 (dddd, 11.3, 11.3, 5.6, 5.6 Hz, 1H, CHOAc), 3.23 (ddd, 14.0, 5.1, 1.0 Hz, 1H, 4α-H), 2.19 (dd, 14.2, 11.6 Hz, 1H, 4β-H), 2.00-2.18 (m, 3H, CH, CH_2), 2.01 (s, 3H, CH_3CO_2), 1.72-1.89 (m, 3H, CH, CH_2), 1.00-1.70 (m, 15H, CH, CH_2), 0.984 (s, 3H, CH_3-19), 0.936 (d, 6.6 Hz, 3H, CH_3-21), 0.855 (d, 6.6 Hz, 3H, CH_3-26), 0.850 (d, 6.5 Hz, CH_3-27), 0.801 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.02 (wk, CO_2), 149.13 and 146.62 (wk, 2 X CON). 135.24, 129.36 and 127.77

(CH=CH, *para* -ArC), 131.91 (wk, *ipso* -ArC), 128.81 and 126.36 (4C, *ortho* -ArC, *meta* -ArC), 70.62 (CHOAc), 65.43, 65.07, 55.22, 52.90, 49.37, 44.14, 41.10, 39.53, 38.37, 35.92, 35.32, 33.76, 31.07, 28.03, 27.51, 26.04, 23.73, 23.32, 22.84, 22.61, 22.54, 21.30, 19.05, 17.51, 13.03; UV (EtOH) λ_{max} (log ε) 216 (infl., 4.10), 254 (infl., 3.73); MS (NH_3-CI) m/e 618 ($[M + NH_3]^+$, 0.2%), 365 (MH^+ - AcOH - **24**). Anal. calcd for $C_{37}H_{51}N_3O_4$: C, 73.84; H, 8.54; N, 6.98. Found: C, 73.84; H, 8.68; N, 4.95. Preparative TLC (EtOAc 4%/hexane 96%) of recovered sterol acetates allowed isolation of unreacted **5** (4.5%) which was identical to authentic material by IR, ^{1}H NMR and TLC; all other products were intractable mixtures.

6,7-Dihydro-(3β-acetoxycholesta-5,7-diene N-phenyltriazoline-3,5-dione adduct) (27)[256] and N-cyclohexyl analogue (28)

A modification of the procedure of Barton *et al*[256] was used. Adduct **26** (10.9 mg, 18.1 μmol), platinum dioxide (1 mg) and EtOAc (5 mL) were stirred under an atmosphere of hydrogen for 16 h. The mixture was filtered through a pad of silica (1 g) which was thoroughly washed (EtOAc, 25 mL). The filtrates were concentrated *in vacuo* to afford 10.7 mg (98%) of an inseparable mixture of **27** and **28** as a tan oil: IR ($CHCl_3$ cast) 2951, 2932, 2863 (C-H), 1750 (C=O amide), 1735 (C=O ester), 1696 (C=O amide), 1403, 1245, 1052, 752 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) For **27**: δ 7.48 (m, 5H, ArH), 5.62 (m, 1H, CHOAc), 2.80 (ddd, 14.1, 5.5, 1.0 Hz, 1H, 4α-H), 1.0-2.4 (m, 22H, CH, CH_2), 1.99 (s, 3H, CH_3CO_2), 1.120 (s, 3H, CH_3-19), 0.911 (d, 6.2 Hz, 3H, CH_3-21), 0.853 (d, 6.8 Hz, 3H, CH_3-26), 0.848 (d, 6.8 Hz, 3H, CH_3-27), 0.831 (s, 3H, CH_3-18). For **28**: δ 5.62 (m, 1H, CHOAc), 3.92 (m, 1H, CHN), 2.71 (ddd, 14.1, 5.3, 1.0 Hz, 1H, 4α-H), 1.0-2.4 (m, 33H, CH, CH_2), 2.02 (s, 3H, CH_3CO_2), 1.071 (s, 3H, CH_3-19), 0.904 (d, 6.2 Hz, 3H, CH_3-21), 0.853 (d, 6.8 Hz, 3H, CH_3-26), 0.848 (d, 6.8 Hz, 3H, CH_3-27), 0.804 (s, 3H, CH_3-18). The ratio of **27**:**28** from integration was 75:25; ^{13}C NMR (100 MHz, $CDCl_3$) revealed a mixture of 2 compounds in a ratio of ca.

75:25: for **27**: δ 169.78 (C=O ester), 146.92 and 145.40 (wk, C=O amide), 128.77 and 126.57 (4C, *ortho*-ArC, *meta*-ArC), 131.90 (wk, *ipso*-ArC), 127.65 (*para*-ArC), 71.04 (CHOAc), 64.84, 63.27, 55.98, 53.68, 50.58, 43.68, 39.53, 38.65, 38.30, 35.80, 35.48, 35.10, 33.51, 31.64, 28.03, 27.10, 25.42, 23.72, 23.24, 23.12, 22.75, 22.53, 21.21, 21.09, 18.78, 17.60, 14.07; exact mass: for **28**, 609.4489 (609.4505 calcd for $C_{37}H_{59}N_3O_4$, 1%). For **27**, 603.4021 (603.4036 calcd for $C_{37}H_{53}N_3O_4$, 4%).

Cholesta-5,7-dien-3β-ol (6)[241]

A modification of the method of Barton *et al* [253] was used. Adduct **26** (2.48 g, 4.12 mmol) was dissolved in THF (200 mL), and lithium aluminium hydride (4.82 g, 126 mmol) was added with caution. The mixture was heated at reflux for 16 h, cooled, and EtOAc (20 mL) then H_2O (20 mL) were added dropwise to destroy excess hydride. $MgSO_4$ (30 g) was added, and the mixture was stirred for 10 min, and filtered through a pad of anhydrous $MgSO_4$. The filter pad was washed with Et_2O (1 L), and the combined filtrates were concentrated *in vacuo*. Column chromatography ($CHCl_3$) afforded 1.26 g (80%) of white crystalline **6**, which was recrystallised from acetone: mp 138 - 139 °C (anhydrous), (lit.[241] mp 145 - 147.5 °C); $[\alpha]_D^{24}$ -117.1° (*c* 1.88, $CHCl_3$), (lit.[241] $[\alpha]_D^{30.5}$ -120° (*c* 0.70, $CHCl_3$)); IR ($CHCl_3$ cast) 3350 (br, O-H), 2952, 2931, 2868 (C-H), 1464, 1365, 1064, 1039 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.58 (AB, 5.7 Hz, d, 2.4 Hz, 1H, 6-CH=C), 5.39 (AB, 5.4 Hz, dd, 2.7, 2.7 Hz, 1H, 7-CH=C), 3.64 (m, 1H, CHOH), 2.46 (AB, 13.4 Hz, d, 12 Hz, m, ≤ 2 Hz, 4β-H), 1.0-2.25 (m, 23H, CH, CH_2), 0.945 (s, 3H, CH_3-19), 0.940 (d, 6.2 Hz, 3H, CH_3-21), 0.868 (2 X d, 6.3 Hz, 6H, CH_3-26, CH_3-27), 0.809 (s, 3H, CH_3-18): given assignments were based on ^1H-^1H decoupling experiments; ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.42 and 139.86 (wk, C=CH-CH=C), 119.71 and 116.40 (C=CH-CH=C), 70.50 (CHOH), 56.05, 54.59, 46.37, 43.01, 40.89, 39.58, 39.32, 38.47, 37.09, 36.20, 36.18, 32.09, 28.12, 28.04, 23.95, 23.07, 22.82, 22.58, 21.20, 18.90, 16.33, 11.85; UV (EtOH) λ_{max} (log ε) 253 (infl.,

3.84), 262 (infl., 4.08), 270 (4.22), 281 (4.25), 293 (4.02), (lit.[241] 272 (4.05), 282 (4.08), 293 (3.82)); exact mass: 384.3388 (384.3392 calcd for $C_{27}H_{44}O$). Anal. calcd for $C_{27}H_{44}O$: C, 84.38; H, 11.46. Found: C, 84.30; H, 11.33.

(22*E*)-3β-Acetoxyergosta-5,7,22-triene (29)[387, 388]

The method for conversion of **3** to **5** was used. Ergosterol (26.2 g, 66.0 mmol) afforded 24.1 g (83%) of crystalline **29**: mp 171.5 - 172.5 °C, (lit.[387] mp 181 °C); $[\alpha]_D^{24}$ -87.7° (*c* 1.00, $CHCl_3$), (lit.[387] $[\alpha]_D^{24}$ -90 ± 3°); IR ($CHCl_3$ cast) 2960, 2870 (C-H), 1735 (C=O), 1255 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.58 (dd, 5.6, 2.4 Hz, 1H, C<u>H</u>=C), 5.40 (ddd, 5.2, 2.6, 2.6 Hz, 1H, C<u>H</u>=C), 5.20 (m, 2H, C<u>H</u>=C<u>H</u>), 4.70 (m, 1H, C<u>H</u>OAc), 2.50 (A<u>B</u>, 14.8 Hz, dd, 5.2, 2.4 Hz, 1H, 4α-<u>H</u>), 2.38 (A<u>B</u>, 12.8 Hz, br, 4β-<u>H</u>), 2.02 (s, 3H, $C\underline{H}_3CO_2$), 1.2-2.2 (m, 18H, C<u>H</u>, $C\underline{H}_2$), 1.035 (d, 6.8 Hz, 3H, $C\underline{H}_3$), 0.949 (s, 3H, $C\underline{H}_3$-19), 0.914 (d, 6.8 Hz, 3H, $C\underline{H}_3$), 0.832 (d, 6.8 Hz, 3H, $C\underline{H}_3$), 0.818 (d, 6.8 Hz, 3H, $C\underline{H}_3$), 0.615 (s, 3H, $C\underline{H}_3$-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.49 (wk, $\underline{C}O_2$), 141.52 and 138.63 (wk, <u>C</u>-5, <u>C</u>-8), 135.65, 132.11, 120.31 and 116.42 (<u>C</u>-6, <u>C</u>-7, <u>C</u>-22, <u>C</u>-23), 72.85 (<u>C</u>HOAc), 55.86, 54.60, 46.15, 42.89, 42.89, 40.42, 39.13, 37.99, 37.17, 36.72, 33.15, 28.28, 28.18, 23.04, 21.37, 21.14, 21.09, 19.97, 19.68, 17.63, 16.21, 12.09; MS (EI) m/e 378 (M^+ - HOAc, 100%).

(22*E*)-3β-Acetoxy-5α-hydroxy-6-oxoergosta-7,22-diene (30)[263]

A modification[264] of the procedure of Burawoy[263] was used. To a suspension of **29** (5.13 g, 11.7 mmol) in glacial AcOH (100 mL) was added a solution of chromium trioxide (3.63 g, 36 mmol) in AcOH 80%/H_2O 20% (20 mL). The mixture was stirred for 16 h, methanol (1 mL) was added, and the mixture was poured into H_2O (500 mL). The mixture was filtered through celite, which was washed (H_2O, 100 mL), and dried *in vacuo*. The celite pad was extracted with boiling EtOAc (2 X 250 mL), and the whole filtered. The filtrate was concentrated to 30 mL, and slowly cooled to 0 °C. The crystals

were collected, washed with cold EtOAc (5 mL), and dried to give 1.37 g (25%) of a white crystalline material that was homogeneous by TLC in several different solvent systems, and whose physical constants agreed with those reported for proposed structure **30**; however, ^{13}C NMR showed it to be an inseparable mixture of two compounds: mp 264 - 266 °C dec., (lit.[264] mp 263 - 265 °C dec.); $[\alpha]_D^{24}$ -0.6° (*c* 0.99, $CHCl_3$), (lit.[264] $[\alpha]_D$ -5° (*c* 1, $CHCl_3$)); IR ($CHCl_3$ cast) 3410 (O-H), 2955, 2870 (C-H), 1735 (C=O ester), 1680 (C=O), 1248 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.64 (br s, 1H, C=C<u>H</u>-C=O), 5.23, 5.18 (AB, 16 Hz, d, 8 Hz, 2H, C<u>H</u>=C<u>H</u>), 5.05 (m, 1H, C<u>H</u>OAc), 2.58 (m, 1H, C<u>H</u>, $C\underline{H}_2$), 2.42 (br s, 1H, O<u>H</u>), 1.2-2.25 (m, 18H, C<u>H</u>, $C\underline{H}_2$), 2.02 (s, 3H, $C\underline{H}_3CO_2$), 1.07 (d, 7 Hz, 3H, $C\underline{H}_3$), 1.00 (s, 3H, $C\underline{H}_3$-19), 0.95 (d, 7 Hz, 3H, $C\underline{H}_3$), 0.89 (d, 7 Hz, 3H, $C\underline{H}_3$), 0.86 (d, 7 Hz, 3H, $C\underline{H}_3$), 0.64 (s, 3H, $C\underline{H}_3$-18); MS (NH_3-CI) m/e 488 ([M + NH_3]$^+$); UV (EtOH) λ_{max} 252 nm.

(22*E*)-3β-Acetoxyergosta-5,7,22-triene N-phenyltriazoline-3,5-dione adduct (31)[253]

The method of Barton *et al*[253] was used. A solution of **29** (1.04 g, 2.37 mmol) in $CHCl_3$ (10 mL) was added to a solution of **24** (420 mg, 2.40 mmol) in dry acetone (40 mL) at -78 °C. The mixture was stirred for 3 h at -78 °C, then alumina (Woelm, grade V, 2 g) was added, and the mixture was allowed to warm to room temperature. The mixture was filtered, and the alumina washed (acetone, 20 mL). The combined filtrates were concentrated *in vacuo*. Column chromatography of the resulting glass ($CHCl_3$, then MeOH 2%/$CHCl_3$ 98%) afforded:

Fraction (1), recovered **29** (8%).

Fraction (2), 1.23 g (84%) of **31**: mp 112 - 113 °C; $[\alpha]_D^{24}$ -116.4° (*c* 0.94, $CHCl_3$), (lit.[253] $[\alpha]_D^{24}$ -118° (*c* 0.98, $CHCl_3$)); IR ($CHCl_3$ cast) 2955, 2870 (C-H), 1753 (C=O amide), 1733 (C=O ester), 1703 (C=O amide), 1398, 1242, 755 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.46, 7.30 (m, 5H, Ar<u>H</u>), 6.44, 6.27 (AB, 8.8 Hz, 2H,

CH=CH (ring)), 5.49 (dddd, 11.2, 11.2, 5.6, 5.6 Hz, 1H, CHOAc), 5.23 (m, 2H, CH=CH (chain)), 3.25 (dd, 14.4, 4.8 Hz, 1H, 4α-H), 2.50 (m, 1H, CH, CH_2), 2.35 (m, 1H, CH, CH_2), 2.04-2.26 (m, 4H, CH, CH_2), 2.03 (s, 3H, CH_3CO_2), 1.71-1.93 (m, 5H, CH, CH_2), 1.22-1.71 (m, 8H, CH, CH_2), 1.03 (d, 8 Hz, 3H, CH_3), 0.98 (s, 3H, CH_3-19), 0.91 (d, 7 Hz, 3H, CH_3), 0.72-0.86 (2 X d and s, 9H, 3 X CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.81 (C=O ester), 149.16 and 146.50 (2 X C=O amide), 135.12, 135.05, 132.46, 129.28 and 127.64 (CH=CH (ring), CH=CH (chain), *para* -ArC), 131.92 (wk, *ipso* -ArC), 128.76 and 126.19 (4C, *ortho* -ArC, *meta* -ArC), 70.54 (CHOAc), 65.37 and 64.92 (2 X C-N), 55.27, 52.94, 49.36, 43.92, 42.87, 41.17, 39.40, 38.11, 33.63, 33.18, 31.04, 27.42, 25.96, 23.34, 22.42, 21.20, 21.20, 19.96, 19.64, 17.52, 17.40, 13.31; UV (MeOH) λ_{max} (log ε) 257 (3.53), (lit.[253] 255 (3.63)); MS (NH_3-CI) m/e 613 (M^+, 0.6%), 378 (M^+ - AcOH - **24**, 100%). Anal. calcd for $C_{38}H_{51}N_3O_4$: C, 74.35; H, 8.38; N, 6.85. Found: C, 74.63; H, 8.48; N, 6.78.

6,7-Dihydro-(3β-acetoxyergosta-5,7-diene N-phenyltriazoline-3,5-dione adduct) (32)[256]

The procedure[256] used to convert **26** to **27** was used. Thus, **31** (46.7 mg, 76.0 μmol) afforded 45.3 mg (96%) of **32** as an oil: $[\alpha]_D^{24}$ -91.3° (*c* 2.00, $CHCl_3$); IR ($CHCl_3$ cast) 2950, 2865 (C-H), 1750 (C=O amide), 1732 (C=O ester), 1698 (C=O amide), 1400, 1240 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.48 (m, 2H, ArH), 7.42 (t, 7.2 Hz, 2H, ArH), 7.32 (m, 1H, ArH), 5.63 (dddd, 10.8, 10.8, 5.4, 5.4 Hz, 1H, CHOAc), 2.80 (dd, 14.0, 4.8 Hz, 1H, 4α-H), 2.25-2.45 (m, 2H, CH, CH_2), 2.00 (s, 3H, CH_3CO_2), 1.0-2.9 (m, 26H, CH, CH_2), 1.112 (s, 3H, CH_3-19), 0.916 (d, 6.0 Hz, 3H, CH_3), 0.837 (d, obscure, 3H, CH_3), 0.825 (s, 3H, CH_3-18), 0.762 (d, 6.8 Hz, 3H, CH_3), 0.755 (d, 6.8 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.22 (wk, C=O ester), 147.02 and 145.51 (wk 2 X C=O amide), 132.09 (wk, *ipso* -ArC), 128.96 and 126.72 (4C, *ortho* -ArC, *meta* -ArC), 127.84 (*para* -ArC), 70.89 (CHOAc), 64.65 and

63.09 (2 X C-N), 55.46, 53.38, 50.28, 43.35, 38.78, 38.28, 37.90, 35.52, 34.72, 33.12, 33.02, 31.28, 31.21, 30.10, 29.38, 26.70, 25.02, 22.81, 22.75, 20.91, 20.67, 20.15, 18.58, 17.24, 15.02, 13.66; exact mass 617.4164 (617.4192 calcd for $C_{38}H_{55}N_3O_4$). Compound **32** was similarly prepared by catalytic hydrogenation of **31** (10.0 mg) in dioxan 90%/acetic acid 10% with PtO_2 (1 mg)[264] for 16 h (96% yield), or in EtOAc with 5% Pd/C (2 mg)[389] for 2 h (80% yield).

(22*E*)-Ergosta-5,22-dien-3β-ol (8) and (22*E*)-5α,β-ergosta-7,22-dien-3β-ol from 31[265]

The procedure of Anastasia *et al*[265] was used. Lithium (2.80 g, 400 mmol) was added in small portions to a solution of **31** (5.65 g, 9.20 mmol) in refluxing anhydrous ethylamine (30 mL, freshly distilled from Li). The mixture was stirred until it turned blue, then a further 30 min. Solid NH_4Cl was added until the blue colour disappeared, and excess Li was destroyed with 2-propanol (10 mL) then H_2O (200 mL). The mixture was extracted ($CHCl_3$, 3 X 250 mL) and the extracts washed (5% HCl, 375 mL), dried and concentrated *in vacuo*. Column chromatography ($CHCl_3$) afforded 2.60 g (71%) of a mixture of isomers **8** and (22*E*)-5α,β-ergosta-7,22-dien-3β-ol in a ratio of 56:44 by ^1H NMR; TLC in several solvent systems, and on plates impregnated with $AgNO_3$, showed a single spot. Extensive column chromatography gave only partial separation of the isomers. Preparative HPLC also failed to separate these compounds completely, while fractional crystallisation afforded no separation. IR ($CHCl_3$ cast) 3380 (br, O-H), 2854-2950 (C-H) cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 5.35 (m, 1H, CH=C), 5.20 (m, 2H, CH=CH), 3.60 (m, 1H, CHOH), 1.13-2.50 (m, 24H, CH, CH_2), 0.78-1.05 (4 X d, s, 15H, 5 X CH_3), 0.70 (s, 3H, CH_3-18 in **8**), 0.55 (s, 3H, CH_3-18 in (22*E*)-5-*trans*-ergosta-5,22-dien-3β-ol); exact mass: 396.3389 (396.3392 calcd for $C_{28}H_{44}O$).

(22*E*)-Ergosta-5,22-dien-3β-ol (8) and (22*E*)-5α,β-ergosta-7,22-dien-3β-ol from ergosterol (4)[266]

The method of Barton *et al*[266] was used. Ergosterol (77.2 g, 195 mmol) was dissolved in HMPA (1 L, freshly distilled from Na) and THF (2 L). The mixture was cooled in ice-water, and a solution of n-butyllithium (1.55 M in hexane, 133 mL, 200 mmol) was added. Lithium (8.23 g, 1.18 mol) was added in small pieces, followed immediately by anhydrous di-isopropylamine (84.5 mL, 600 mmol). The mixture was stirred, and a blue color developed after 5 h. Excess Li was destroyed with NH_4Cl (100 g) then H_2O (100 mL). Most of the THF was removed on the rotary evaporator, and the residue diluted (H_2O, 2 L), and extracted (benzene, 5 X 500 mL). The combined organic phases were washed (H_2O, 1 L), dried and concentrated *in vacuo*. Column chromatography ($CHCl_3$) afforded 60.6 g (78%) of a mixture of **8** and (22*E*)-5α,β-ergosta-5,22-dien-3β-ol as an off-white solid.

(22*E*)-3β-(*p*-Toluenesulfonoxy)-ergosta-5,22-diene (33)[267] and (22*E*)-3β-(*p*-toluenesulfonoxy)-5-*trans*-ergosta-7,22-diene (34)[266, 390]

The procedure of Barton *et al*[266] was used. A mixture of alcohols from reduction of ergosterol (99.2 mg, 249 μmol), *p*-toluenesulfonyl chloride (200 mg, 1.05 mmol) and pyridine (2 mL) was stirred at room temperature for 20 h. H_2O (25 mL) was added, and the mixture was extracted (2 X CH_2Cl_2, 25 mL). The extracts were washed (H_2O, 30 mL), dried and concentrated *in vacuo*. Filtration through silica and elution with $CHCl_3$ afforded 123 mg (89%) of a mixture of **33** and **34** as a white solid: ^1H NMR (80 MHz, $CDCl_3$) δ 7.82, 7.35 (AB, 8 Hz, 4H, ArH), 5.20 (m, 3H, C=CH, CH=CH), 4.40 (m, 1H, CHOTs), 2.40 (s, 3H, $ArCH_3$), 1.05-2.5 (m, 23H, CH, CH_2), 0.73-0.98 (m, 15H, 5 X CH_3), 0.61 (s, CH_3-18 of **33**), 0.46 (s, CH_3-18 of **34**); exact mass: 552.3627 (552.3637 calcd for $C_{35}H_{52}SO_3$).

(22*E*)-6β-Hydroxy-3α,5-cyclo-5α-ergost-22-ene (35)[266]

The procedure of Thompson *et al*[267] was used. To a solution of potassium carbonate (104 mg, 188 μmol) in H_2O (13.5 mL) and acetone (31.5 mL) at reflux, was added a solution of **33** and **34** (125 mg, 227 μmol) in acetone (7 mL) over 5 min. The mixture was heated to reflux for 15 min, then distilled until 30 mL of distillate had been collected. The residue was cooled and extracted (Et_2O, 2 X 50 mL). The extracts were washed (H_2O, 2 X 100 mL), dried and concentrated *in vacuo*. Column chromatography (benzene) afforded:

Fraction (1) 76.4 mg (61%) of unreacted **34**, which was recrystallised from acetone to give pure *trans*-tosylate (**34**)[390] as white crystals: mp 141.5 - 142.5 °C dec.; $[\alpha]_D^{24}$ -22.2° (*c* 2.27, $CHCl_3$); IR ($CHCl_3$ cast) 3020 (Ar-H), 2954, 2928, 2892, 2868, 2845 (C-H), 1600, 1471 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.83, 7.36 (AB, 8.0 Hz, 4H, ArH), 5.22 (m, 2H, CH=CH), 5.15 (br m, 1H, CH=C), 4.45 (dddd, 11.2, 11.2, 4.8, 4.8 Hz, 1H, CHOTs), 2.45 (s, 3H, $ArCH_3$), 1.05-2.00 (m, 23H, CH, CH_2), 1.002 (d, 6.5 Hz, 3H, CH_3), 0.906 (d, 6.7 Hz, 3H, CH_3), 0.830 (d, 6.5 Hz, 3H, CH_3), 0.812 (d, 6.5 Hz, 3H, CH_3), 0.761 (s, 3H, CH_3-19), 0.505 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.31, 139.56, 135.72 and 135.20 (wk, ArCMe, ArCS, C-5, C-8), 129.84 and 127.72 (4C, 2 X ArCH), 132.13 and 117.24 (C-6, C-7), 82.21 (CHOTs), 56.23, 55.18, 49.27, 43.44, 42.90, 40.54, 40.43, 39.58, 37.04, 34.73, 34.16, 33.20, 29.54, 28.61, 28.03, 23.07, 21.62, 21.62, 21.20, 20.05, 19.73, 17.68, 12.87, 12.12; exact mass: 552.3638 (552.3639 calcd for $C_{35}H_{52}SO_3$). Anal. calcd for $C_{35}H_{52}SO_3$: C, 76.04; H, 9.48; S, 5.80. Found: C, 76.00; H, 9.22; S, 5.87.

Fraction (2), 26.5 mg (29%) of **35** as a white solid: mp 115 - 116 °C, (lit.[267] mp 113 - 115 °C); $[\alpha]_D^{24}$ +14.5° (*c* 2.06, $CHCl_3$), (lit.[267] $[\alpha]_D^{24}$ +15° (1%, $CHCl_3$)); IR ($CHCl_3$ cast) 3420 (br, O-H), 2955, 2931, 2906, 2858 (C-H), 1455 (wk), 1030 (wk), 965 (wk) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.20 (m, 2H, CH=CH), 3.28 (dd, 2.4, 2.4 Hz, 6α-H), 1.99 (dddd, 6.4, 6.4, 6.4, 2.4 Hz, 1H, CH), 1.94 (ddd, 12.8, 3.2, 3.2

Hz, 1H, CH), 1.1-1.95 (m, 22H, CH, CH_2), 1.001 (s, 3H, CH_3-19), 0.985 (br d, 6.6 Hz, 3H, CH_3), 0.890 (m, 1H, 3-H), 0.886 (d, 6.8 Hz, 3H, CH_3), 0.810 (d, 6.8 Hz, 3H, CH_3), 0.793 (d, 6.0 Hz, 3H, CH_3), 0.710 (s, 3H, CH_3-18), 0.50 (dd, 4.2, 4.2 Hz, 1H, 4-H), 0.26 (dd, 8.0, 4.8 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 135.97 and 131.82 (CH=CH), 73.84 (CHOH), 56.65, 56.24, 47.71, 42.98, 42.87, 42.68, 40.14, 40.14, 38.96, 37.21, 33.26, 33.15, 29.92, 28.63, 25.07, 24.24, 24.19, 22.73, 20.91, 20.24, 19.96, 19.63, 17.61, 12.48, 11.60; exact mass: 398.3557 (398.3566 calcd for $C_{28}H_{46}O$). Anal. calcd for $C_{28}H_{46}O$: C, 84.36; H, 11.63. Found: C, 84.35; H, 11.65.

Fraction (3), (22*E*)-Ergosta-5,22-dien-3β-ol (8) which was contaminated with other compounds, and was not readily purified.

(22*E*)-Ergosta-5,22-dien-3β-ol (8)[267]

The procedure of Thompson *et al*[267] was used. A mixture of 35 (2.22 g, 5.57 mmol), *p* -toluenesulfonic acid (0.50 g, 2.9 mmol), dioxan (175 mL) and H_2O (175 mL) was heated at 70 °C for 16 h. The mixture was cooled, diluted with H_2O (200 mL) and extracted ($CHCl_3$, 2 X 200 mL). The extracts were washed (H_2O, 200 mL), dried, and concentrated *in vacuo* . Column chromatography ($CHCl_3$) afforded 2.02 g (91%) of white crystalline 8, which was recrystallised from acetone to give long needles: mp 145 - 146 °C, (lit.[267] mp 148 °C); $[\alpha]_D^{24}$ -63.0° (*c* 2.05, $CHCl_3$), (lit.[267] $[\alpha]_D^{24}$ -66° ($CHCl_3$)); IR ($CHCl_3$ cast) 3340 (br, O-H), 2954, 2934, 2891, 2869 (C-H), 1453, 1380, 1367, 1059, 966, 958 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.38 (br ddd, 2.6, ≈1, ≈1 Hz, 1H, CH=C), 5.20 (m, 2H, CH=CH), 3.515 (dddd, 11.4, 11.4, 4.4, 4.4 Hz, 1H, CHOH), 2.21 (m, 2H, CH, CH_2), 1.93 (m, 3H, CH, CH_2), 1.79 (m, 3H, CH, CH_2), 0.9-1.7 (m, 16H, CH, CH_2), 1.010 (d, 6.4 Hz, 3H, CH_3), 1.006 (s, 3H, CH_3-19), 0.907 (d, 6.8 Hz, 3H, CH_3), 0.838 (d, 6.4 Hz, 3H, CH_3), 0.816 (d, 6.8 Hz, 3H, CH_3), 0.689 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.94 (wk, C=CH), 135.92, 131.87 and 121.71 (C=CH, CH=CH), 71.81 (CHOH), 56.97, 56.12, 50.34, 42.96,

42.40, 42.31, 40.17, 39.84, 37.32, 36.61, 33.17, 31.98, 31.96, 31.74, 28.55, 24.37, 21.10, 21.08, 20.04, 19.76, 19.43, 17.62, 12.14; exact mass: 398.3546 (398.3549 calcd for $C_{28}H_{46}O$). Anal. calcd for $C_{28}H_{46}O$: C, 84.36; H, 11.63. Found: C, 84.26; H, 11.48.

6β-Hydroxy-3α,5-cyclo-5α-ergostane (36)[267] and 6β-hydroxy-5β-methyl-4-nor-ergostane (37)

The method[256] for conversion of **26** to **27** was used. Thus, **35** (11.55 g, 30.0 mmol) afforded 11.33 g (98%) of **36**, contaminated with 5 - 10% of **37**, as a chromatographically inseparable oil: $[\alpha]_D^{24}$ +36.0° (*c* 2.33, $CHCl_3$); IR ($CHCl_3$ cast) 3420 (O-H), 2955, 2932, 2858 (C-H), 1462, 1375, 1017 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 3.26 (dd, 2.6, 2.6 Hz, 1H, CHOH), 1.0-2.2 (m, 26H, CH, CH_2), 1.056 (s, 3H, CH_3-19), 0.918 (d, 6.4 Hz, 3H, CH_3), 0.857 (d, 6.8 Hz, 3H, CH_3), 0.785 (d, 6.8 Hz, 3H, CH_3), 0.779 (d, 6.5 Hz, 3H, CH_3), 0.723 (s, 3H, CH_3-18), 0.518 (dd, 4.2, 4.2 Hz, 1H, 4-H), 0.290 (dd, 8.0, 5.0 Hz, 1H, 4-H); exact mass: 400.3691 (400.3705 calcd for $C_{28}H_{48}O$). Anal. calcd for $C_{28}H_{48}O$: C, 83.93; H, 12.07. Found: C, 83.80; H, 12.19.

Ergost-5-en-3β-ol (22)[267, 391]

The method[267] for conversion of **35** to **8** was used. Thus **36** (containing 5-10% of **37**, 2.36 g, 5.90 mmol) gave, after column chromatography ($CHCl_3$):

Fraction (1), crude **37**; repeated column chromatography (EtOAc 4%/hexane 96%) afforded 156 mg (7%) of **37** as a gum: $[\alpha]_D^{24}$ +26.6° (*c* 1.22, $CHCl_3$); IR ($CHCl_3$ cast) 3460 (br, O-H), 2952, 2868 (C-H), 1462 (wk), 1375 (wk) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.70 (dd, 3.0, 2.4 Hz, 1H, CHOH), 2.00 (ddd, 12.4, 3.0, 3.0 Hz, 1H, 7β-H), 1.00-1.90 (m, 27H, CH, CH_2), 1.020 (s, 3H, CH_3), 0.956 (s, 3H, CH_3), 0.890 (d, 6.6 Hz, 3H, CH_3), 0.846 (d, 7.2 Hz, 3H, CH_3), 0.787 (d, 6.8 Hz, 3H, CH_3), 0.692 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$, multiplicities determined by INEPT and APT phase-

selective spectra) δ 73.48 (CHOH), 56.91 (CH), 56.42 (CH), 48.27 (C), 45.58 (C), 45.32 (C), 42.87 (C), 40.52 (CH_2), 39.20 (CH), 36.46 (CH_2), 36.33 (CH), 36.27 (CH_2), 35.01 (CH_2), 33.97 (CH_2), 31.60 (CH), 30.74 (CH_2), 30.05 (CH), 28.42 (CH_2), 24.31 (CH_2), 22.60 (CH_2), 22.04 (CH_2), 22.04 (CH_3), 20.57 (CH_3), 19.24 (CH_2), 19.04 (CH_3), 17.87 (CH_3), 17.32 (CH_3), 15.66 (CH_3), 12.38 (CH_3); exact mass: 402.3851 (402.3862 calcd for $C_{28}H_{50}O$). Anal. calcd for $C_{28}H_{50}O$: C, 83.51; H, 12.52. Found: C, 83.27; H, 12.13.

Fraction (2), 1.81 g (82%) of white crystalline **22**, which was recrystallised from acetone, mp 154 - 155 °C, (lit.[391] mp 157 - 158 °C); $[\alpha]_D^{24}$ -47.2° (*c* 1.92, $CHCl_3$), (lit.[391] $[\alpha]_D^{24}$ -46.3° ($CHCl_3$)); IR ($CHCl_3$ cast) 3360 (br, O-H), 2958, 2933, 2906, 2870 (C-H), 1462, 1375, 1060 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.30 (d, 5.2 Hz, m, 1H, CH=C), 3.46 (dddd, 11.0, 11.0, 4.4, 4.4 Hz, 1H, CHOH), 2.20 (m, 2H, CH_2), 1.93 (m, 2H, CH, CH_2), 1.79 (m, 4H, CH, CH_2), 0.70-1.56 (m, 20H, CH, CH_2), 1.001 (s, 3H, CH_3-19), 0.917 (d, 6.4 Hz, 3H, CH_3), 0.854 (d, 6.8 Hz, 3H, CH_3), 0.780 (d, 6.8 Hz, 3H, CH_3), 0.772 (d, 6.8 Hz, 3H, CH_3), 0.672 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.95 (wk, C=CH), 121.84 (CH=C), 71.97 (CHOH), 57.02, 56.35, 50.41, 42.50, 40.03, 39.35, 37.42, 36.78, 36.33, 33.92, 32.10, 32.03, 32.03, 31.94, 31.77, 30.80, 28.22, 24.43, 21.27, 20.54, 19.42, 19.07, 17.88, 15.61, 11.98; exact mass: 400.3705 (400.3705 calcd for $C_{28}H_{48}O$). Anal. calcd for $C_{28}H_{48}O$: C, 83.93; H, 12.07. Found: C, 83.96; H, 12.14.

Crystallization of ergost-5-en-3β-ol (22) for X-ray diffraction

The method of Shieh *et al* [325] was used. Thus, **22** (10 mg) was dissolved in Et_2O (0.5 mL) and EtOH (0.5 mL) was added. The solution was placed in a small tube (4 mm i.d.) and the tube inserted into a test-tube containing EtOH 50%/H_2O 50%. The test-tube was sealed, and stored for 2 weeks. Solvent was decanted to give long thin needles of **22**.

(22*E*)-3β-Benzoyloxyergosta-5,7,22-triene (38)[281]

A modification of the procedure of Klosty *et al*[281] was used. Benzoyl chloride (4.45 g, 31.7 mmol) was added to a solution of ergosterol (3.97 g, 10.0 mmol) and pyridine (2 mL) in CH_2Cl_2 (100 mL) at 0 °C. The mixture was heated to reflux for 1 h. Water (100 mL) was added, and the organic phases were washed (10% Na_2CO_3, 100 mL, then H_2O, 100 mL), dried, and concentrated *in vacuo*. The residue was recrystallised twice from EtOH to give 2.30 g (46%) of **38** as white needles: mp 153 - 156 °C, (lit.[281] mp 169 °C); $[\alpha]_D^{24}$ -59.4° (*c* 1.00, $CHCl_3$), (lit.[281] $[\alpha]_D$ -72° ($CHCl_3$)); IR ($CHCl_3$ cast) 3030 (Ar-H), 2985, 2860, 2840 (C-H), 1710 (C=O), 1450, 1270, 1115, 695, 685 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, 8.0 Hz, 2H, *ortho*-ArH), 7.52 (t, 7.6 Hz, 1H, *para*-ArH), 7.41 (t, 7.6 Hz, 2H, *meta*-ArH), 5.60 (AB, 4.8 Hz, m, 1H, C=CH), 5.40 (AB, 4.8 Hz, m, 1H, C=CH), 5.20 (m, 2H, CH=CH (chain)), 4.95 (dddd, 11.6, 11.6, 4.0, 4.0 Hz, 1H, CHOBz), 2.62 (AB, 14.4 Hz, dd, 4.8, 2.0 Hz, 1H, 4α-H), 2.50 (AB, 14.4 Hz, m, 1H, 4β-H), 1.1-2.2 (m, 18H, CH, CH_2), 1.070 (d, 6.6 Hz, 3H, CH_3), 1.022 (s, 3H, CH_3-19), 0.947 (d, 6.8 Hz, 3H, CH_3), 0.867 (d, 6.2 Hz, 3H, CH_3), 0.852 (d, 6.8 Hz, 3H, CH_3), 0.655 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.27 (C=O), 140.88, 137.88 and 130.19 (wk, C-5, C-8, *ipso*-ArC), 135.00, 132.23, 131.40, 119.79 and 115.79 (C-6, C-7, C-22, C-23, *para*-ArC), 128.90 and 127.74 (4C, *ortho*-ArC, *meta*-ArC), 72.78 (CHOBz), 55.14, 53.92, 45.49, 42.22, 42.19, 39.74, 38.43, 37.35, 36.55, 36.17, 32.44, 27.62, 27.62, 22.39, 20.54, 20.43, 19.36, 19.06, 17.03, 15.61, 11.47; exact mass: 500.3666 (500.3654 calcd for $C_{35}H_{48}O_2$). Anal. calcd for $C_{35}H_{48}O_2$: C, 83.95; H, 9.66. Found: C, 83.96; H, 9.47.

(22*E*)-3β-Benzoyloxyergosta-5,7,22-triene iron tricarbonyl adduct (39)[256]

The procedure of Barton *et al*[256] was followed. Thus, a mixture of **38** (1.50 g, 3.00 mmol), *p*-methoxybenzylideneacetone (1.70 g, 9.7 mmol) and di-iron nonacarbonyl (3.63 g, 10.0 mmol) in toluene (13 mL) was heated at 55 °C with stirring for 26 days. The

yellow solution was concentrated *in vacuo* and the residue crystallised (EtOAc) to remove 413 mg of unreacted **38**. The mother liquors were concentrated *in vacuo*. Column chromatography (EtOAc 4%/hexane 96%) gave fractions containing **38** and **39**, which were recrystallised (EtOAc 4%/hexane 96%) to afford 525 mg (27%) of **39** as a solid which was free of **38** by TLC (EtOAc 4%/hexane 96%, 2X): mp 147 - 148 °C, (lit.[256] mp 163 - 165 °C); $[\alpha]_D^{24}$ -57.9° (*c* 1.20, $CHCl_3$), (lit.[256] $[\alpha]_D^{24}$ -67.9° (*c* 1.20, $CHCl_3$)); IR ($CHCl_3$ cast) 2950, 2865 (C-H), 2033, 1955 (FeC=O), 1720 (C=O ester), 1450, 1272, 1110, 708 cm^{-1}: no absorption at 1600, 1585 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.04, 7.54 and 7.42 (m, 5H, ArH), 5.23 (d, 4.4 Hz, 1H, CH=C (ring)), 5.20 (AB, 15.4 Hz, d, 7.0 Hz, 1H, CH=CH (chain)), 5.17 (AB, 15.4 Hz, d, 8.0 Hz, 1H, CH=CH (chain)), 5.00 (m, 1H, CHOBz), 4.92 (d, 4.4 Hz, 1H, CH=C (ring)), 2.50 (dd, 13.6, 12.0 Hz, 1H, CH, CH_2), 1.10-2.20 (m, 19H, CH, CH_2), 1.00 (d, 6.5 Hz, 3H, CH_3), 0.965 (s, 3H, CH_3-19), 0.901 (d, 6.8 Hz, 3H, CH_3), 0.835 (d, 6.5 Hz, 3H, CH_3), 0.817 (d, 7.0 Hz, 3H, CH_3), 0.725 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.33 (FeC=O), 164.88 (C=O ester), 134.35, 131.98 and 131.30 (CH=CH (chain), *para* -ArC), 129.84 (wk, *ipso* -ArC), 128.62 and 127.48 (4C, *ortho* -ArC, *meta* -ArC), 87.65 and 80.74 (wk, C-5, C-8), 82.59 and 77.79 (C-6, C-7), 73.09 (CHOBz), 57.13, 55.49, 55.25, 45.56, 41.85, 38.88, 37.57, 37.16, 37.07, 32.12, 27.52, 27.31, 26.86, 26.36, 24.16, 23.26, 20.22, 19.10, 18.77, 16.72, 10.93; MS (EI) m/e 612 (M^+ - CO, 1%), 584 (M^+ - 2 CO, 3%), 556 (M^+ - 3 CO, 27%), 500 (M^+ - $Fe(CO)_3$, 2%), 378 (M^+ - $Fe(CO)_3$ - $PhCO_2H$, 48%).

3β-Acetoxyergost-5-ene (40)[267, 391]

The procedure of Thompson *et al*[267] was used. A mixture of **36** (4.55 g, 11.3 mmol), AcOH (137 mL) and anhydrous zinc acetate (9.50 g, 51.8 mmol) was heated at reflux for 3 h. The mixture was cooled and poured into H_2O (750 mL). The solid was collected and washed (H_2O, 200 mL). Recrystallisation (acetone 50%/ methanol 50%, 250

mL) afforded 3.23 g (64%) of **40** as white plates. Column chromatography (EtOAc 4%/hexane 96%) of the residue gave a further 18% of **40**, total yield 82%, and unreacted **37** (7%). For **40**: mp 144 - 145 °C, (lit.[391] mp 144 - 145 °C); $[\alpha]_D^{24}$ -46.1° (*c* 2.02, $CHCl_3$), (lit.[391] $[\alpha]_D^{24}$ -45.5° (*c* 1.20, $CHCl_3$)); IR ($CHCl_3$ cast) 2956, 2938, 2906, 2871 (C-H), 1731 (C=O), 1465, 1440, 1378, 1252 (str), 1040 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.39 (br d, 4.8 Hz, 1H, C<u>H</u>=C), 4.61 (m, 1H, C<u>H</u>OAc), 2.32 (m, 2H, 4-<u>H</u>$_2$), 2.04 (s, 3H, CH_3CO_2), 2.00 (m, 2H, C<u>H</u>, C<u>H</u>$_2$), 1.86 (m, 3H, C<u>H</u>, C<u>H</u>$_2$), 0.8-1.7 (m, 20H, C<u>H</u>, C<u>H</u>$_2$), 1.016 (s, 3H, CH_3-19), 0.918 (d, 7 Hz, 3H, CH_3), 0.855 (d, 6.8 Hz, 3H, CH_3), 0.783 (d, 6.8 Hz, 3H, CH_3), 0.776 (d, 6.8 Hz, 3H, CH_3), 0.673 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.55 (wk, <u>C</u>=O), 139.87 (wk, <u>C</u>=CH), 122.74 (C=<u>C</u>H), 74.01 (<u>C</u>HOH), 56.85, 56.12, 50.24, 42.40, 39.88, 39.20, 38.27, 37.18, 36.74, 36.20, 33.88, 32.04, 32.04, 31.66, 30.79, 28.26, 27.81, 24.30, 21.44, 21.14, 20.55, 19.38, 18,97, 17.74, 15.55, 11.91; exact mass: 382.3588 (382.3599 calcd for $C_{30}H_{50}O_2$). Anal. calcd for $C_{30}H_{50}O_2$: C, 81.39; H, 11.38. Found: C, 81.33; H, 11.33.

3β-Acetoxyergosta-5,7-diene N-phenyltriazoline-3,5-dione adduct (41)[256]

The method for conversion of **5** to **26** was used. Thus, **40** (3.14 g, 7.09 mmol) gave 2.88 g (66%) of **41** as a pale yellow gum: $[\alpha]_D^{24}$ -80.0° (*c* 2.07, $CHCl_3$), (lit.[256] $[\alpha]_D^{24}$ -96.5° (*c* 0.29, $CHCl_3$)); IR ($CHCl_3$ cast) 2956, 2930 (C-H), 1756 (C=O amide), 1737 (C=O ester), 1706 (C=O amide), 1398, 1242 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.42 (m, 5H, Ar<u>H</u>), 6.43, 6.23 (AB, 8.4 Hz, 2H, C<u>H</u>=C<u>H</u>), 5.46 (dddd, 11.2, 11.2, 5.4, 5.4 Hz, 1H, C<u>H</u>OAc), 3.23 (ddd, 14.0, 5.0, ≈0.6 Hz, 1H, 4α-<u>H</u>), 2.52 (m, 1H, 4β-<u>H</u>), 2.32 (m, 1H, C<u>H</u>, C<u>H</u>$_2$), 1.85-2.23 (m, 4H, C<u>H</u>, C<u>H</u>$_2$), 2.00 (s, 3H, CH_3CO_2), 1.1-1.85 (m, 17H, C<u>H</u>, C<u>H</u>$_2$), 0.985 (s, 3H, CH_3-19), 0.937 (d, 5.8 Hz, 3H, CH_3), 0.847 (d, 6.6 Hz, 3H, CH_3), 0.797 (s, 3H, CH_3-18), 0.772 (d, 7.0 Hz, 6H, 2 X CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.90 (wk, <u>C</u>=O ester), 149.22 and 146.75

(wk, C=O amide), 135.37, 129.38 and 126.31 (CH=CH, *para* -ArC), 132.04 (wk, *ipso* -ArC), 128.87 and 126.33 (4C, *ortho* -ArC, *meta* -ArC), 70.66 (CHOAc), 65.41, 65.12, 55.13, 53.08, 49.44, 44.12, 41.29, 39.18, 38.37, 35.62, 33.84, 33.62, 31.60, 31.17, 30.55, 27.42, 26.01, 23.41, 22.52, 21.28, 20.49, 19.26, 17.83, 17.54, 15.66, 13.01; MS (NH_3-CI) m/e 633 ($[M + NH_4]^+$, 2%), 381 (MH^+ - AcOH - **24**, 100%); UV (MeOH) λ_{max} (log ε) 216 (infl., 4.10), 248 (3.685).

6,7-Dihydro-(3β-acetoxyergosta-5,7-diene N-phenyltriazoline-3,5-dione adduct) (32) from 41

The method[256] used to prepare **27** from **26** was used. Thus, **41** (11.0 mg, 17.9 μmol) afforded 10.8 mg (98%) of **32**, that was identical to material prepared from **31** by IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry.

Ergosta-5,7-dien-3β-ol (21)[256]

The method[253] for conversion of **26** to **6** was used. Thus, **41** (260 mg, 422 μmol) gave 162 mg (96%) of **21** which was recrystallised from acetone as white flakes: mp 134 - 136 °C, (lit.[392] mp 132.5 - 134.5 °C); $[\alpha]_D^{24}$ -121.4° (*c* 2.09, $CHCl_3$), (lit.[256] $[\alpha]_D$ -121° (*c* 0.1, $CHCl_3$)); IR ($CHCl_3$ cast) 3300 (br, O-H), 2953, 2930, 2871 (C-H), 1462, 1376, 1060, 830 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.60 (AB, 5.6 Hz, d, 2.4 Hz, 1H, 6-H), 5.41 (AB, 5.6 Hz, dd, 2.8, 2.8 Hz, 1H, 7-H), 3.65 (dddd, 11.2, 11.2, 4.0, 4.0 Hz, 1H, CHOH), 2.47 (AB, 14.5 Hz, dd, 2.5, 2.0 Hz, 1H, CH_2), 2.28 (AB, 13.0 Hz, br d, 1.8 Hz, 1H, CH_2), 2.08 (ddd, 13.0, 2.5, 2.5 Hz, 1H, CH, CH_2), 1.1-2.0 (m, 22H, CH, CH_2), 0.945 (d, 6.8 Hz, 3H, CH_3), 0.943 (s, 3H, CH_3-19), 0.860 (d, 6.8 Hz, 3H, CH_3), 0.787 (d, 7.4 Hz, 3H, CH_3), 0.782 (d, 7.4 Hz, 3H, CH_3), 0.617 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.44 and 139.96 (wk, 5-C, 8-C), 119.82 and 116.54 (6-C, 7-C), 70.62 (CHOH), 56.15, 54.70, 46.51, 43.18, 41.02, 39.42, 39.38, 38.61, 37.20, 36.77, 33.90, 32.28, 31.77, 30.93, 28.11, 23.18, 21.30,

20.54, 19.16, 17.84, 16.44, 15.68, 11.92; exact mass: 398.3548 (398.3549 calcd for $C_{28}H_{46}O$); UV (MeOH) λ_{max} (log ε) 252 (infl., 3.756), 261 (infl., 4.054), 271 (4.075), 281 (4.095), 293 (3.866), (lit.[256] 262 (3.903), 272 (4.049), 282 (4.072), 294 (3.833)). Anal. calcd for $C_{28}H_{46}O$: C, 84.36; H, 11.63. Found: C, 84.38; H, 11.71.

(22*E*)-3β-*p*-Toluenesulfonoxy-stigmasta-5,22-diene (43)[293, 300]

The method of Steele and Mosettig[300] was used. A mixture of (22*E*)-stigmasta-5,22-dien-3β-ol (74.7 g, 181 mmol), *p*-toluenesulfonyl chloride (73.6 g, 386 mmol) and pyridine (600 mL) was stirred for 16 h. The solution was poured into 10% $KHCO_3$ (1 L) and the precipitate was collected, washed (H_2O, 1 L) and recrystallised (acetone, 3.6 L) to afford 87.9 g (86%) of **43** as white crystals: mp 142 - 143 °C dec., (lit.[293] mp 148 - 149 °C); $[\alpha]_D^{24}$ -50.0° (*c* 1.05, $CHCl_3$), (lit.[293] $[\alpha]_D^{24}$ -50.0°); IR ($CHCl_3$ cast) 2980, 2880 (C-H), 1460, 1362, 1175 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.82, 7.35 (AB, 8.3 Hz, 4H, ArH), 5.31 (d, 4.9 Hz, m, 1H, CH=C), 5.15 (AB, 15.2 Hz, d, 8.6 Hz, 1H, CH=CH), 5.04 (AB, 15.2 Hz, d, 9.0 Hz,1H, CH=CH), 4.32 (dddd, 11.5, 11.5, 4.6, 4.6 Hz, 1H, CHOTs), 2.44 (s, 3H, $ArCH_3$), 2.40 (m, 1H, 4α-H), 2.26 (AB, 13.4 Hz, dd, 5.0, 1.8 Hz, 1H, 4β-H), 0.8-2.1 (m, 21H, CH, CH_2), 1.021 (d, 6.8 Hz, 3H, CH_3), 0.966 (s, 3H, CH_3-19), 0.841 (d, 6.4 Hz, 3H, CH_3), 0.799 (t, 7.2 Hz, 3H, CH_3), 0.790 (d, 7.0 Hz, 3H, CH_3), 0.674 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.30, 139.01 and 135.25 (wk, C=CH, 2 X ArC), 138.21, 129.54 and 123.40 (CH=C, CH=CH), 129.73 and 127.77 (4C, 2 X ArCH), 82.36 (CHOTs), 56.93, 56.18, 51.39, 50.17, 42.38, 40.46, 39.70, 39.02, 37.01, 36.59, 31.91, 31.91, 31.86, 28.83, 28.77, 25.42, 24.40, 21.63, 21.28, 21.17, 21.00, 19.23, 19.16, 12.24, 12.16; exact mass: 394.3596 (394.3599 calcd for $C_{29}H_{46}$, M^+ - TsOH). Anal. calcd for $C_{36}H_{54}SO_3$: C, 76.28; H, 9.60; S, 5.66. Found: C, 76.33; H, 9.62; S, 5.51.

(22*E*)-6β-Methoxy-3α,5-cyclo-5α-stigmast-22-ene (44) and (22*E*)-3β-methoxystigmasta-5,22-diene (45)[293]

The method of Partridge *et al*[293] was used. A mixture of **43** (20.0 g, 35.3 mmol), pyridine (8.5 mL) and MeOH (200 mL) was stirred at reflux for 7 h. The mixture was concentrated *in vacuo*, H_2O (500 mL) was added, and the mixture was extracted (EtOAc, 2 X 500 mL). The combined extracts were dried (Na_2CO_3) and concentrated *in vacuo*. Column chromatography ($CHCl_3$) afforded:

Fraction (1), 9.73 g (65%) of **44** as a clear oil: $[\alpha]_D^{24}$ +28.2° (*c* 2.29, $CHCl_3$), (lit.[293] $[\alpha]_D^{24}$ +34.0° (*c* 1.02, $CHCl_3$)); IR ($CHCl_3$ cast) 2955, 2915, 2860 (C-H), 1095, 1080, 965 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.10 (m, 2H, C<u>H</u>=C<u>H</u>), 3.36 (s, 3H, CH_3O), 2.78 (dd, 2.5, 2.5 Hz, 1H, C<u>H</u>OMe), 1.07-2.15 (m, 23H, C<u>H</u>, CH_2), 1.03 (s, 3H, CH_3-19), 1.02 (d, 7 Hz, 3H, CH_3), 0.85 (d, 6.5 Hz, 3H, CH_3), 0.82 (d, 7 Hz, 3H, CH_3), 0.80 (t, 7 Hz, 3H, CH_3CH_2), 0.74 (s, 3H, CH_3-18); ^{13}C NMR (50 MHz, $CDCl_3$) δ 138.40 and 129.36 (<u>C</u>H=<u>C</u>H), 82.57 (<u>C</u>HOMe), 56.71, 56.65, 56.20, 51.31, 48.20, 43.57, 42.76, 40.64, 40.33, 35.41, 35.28, 33.49, 31.93, 30.60, 29.01, 25.44, 25.07, 24.39, 22.86, 21.53, 21.32, 21.17, 19.35, 19.06, 13.15, 12.54, 12.38; exact mass: 426.3850 (426.3861 calcd for $C_{30}H_{50}O$).

Fraction (2), 1.17 g (8%) of **45** as a gum: $[\alpha]_D^{24}$ -52.0° (*c* 0.96, $CHCl_3$), (lit.[293] $[\alpha]_D^{24}$ -55.2° (*c* 1.16, $CHCl_3$)); IR ($CHCl_3$ cast) 2960, 2940, 2870 (C-H), 1115, 1100, 970 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 5.37 (m, 1H, C<u>H</u>=C), 5.12 (m, 2H, C<u>H</u>=C<u>H</u>), 3.37 (s, 3H, CH_3O), 3.10 (m, 1H, C<u>H</u>OMe), 1.12-2.50 (m, 24H, C<u>H</u>, CH_2), 0.65-1.12 (sh m, 18H, 6 X CH_3); exact mass: 426.3859 (426.3861 calcd for $C_{30}H_{50}O$).

(20*S*)-20-Hydroxymethyl-6β-methoxy-3α,5-cyclo-5α-pregnane (46)[293]

The method of Partridge *et al*[293] was used. A solution of **44** (2.00 g, 4.69 mmol) in CH_2Cl_2 (40 mL) and pyridine (0.4 mL) was subjected to ozonolysis (ca. 1.2 mol.

equiv. O_3) at -78 °C, until a blue color appeared. The reaction vessel was flushed with Ar, and a solution of sodium *bis*-(2-methoxyethoxy)aluminium hydride (Red-Al™) (7.1 M, 1.6 mL, 11.4 mmol) was added. The mixture was stirred for 1 h at -78 °C, warmed to room temperature over 1 h, and 2 N H_2SO_4 (25 mL) was added with cooling. The mixture was extracted (EtOAc, 2 X 100 mL), and the extracts were dried and concentrated *in vacuo*. Column chromatography over florisil (packed in pyridine 1%/benzene 99%, eluted with benzene, then Et_2O 5%/benzene 95%) afforded 1.00 g (62%) of **46** as a fluffy solid: mp 46 - 48 °C (sealed tube), (lit.[293] glass); $[\alpha]_D^{24}$ +45.7° (*c* 1.04, $CHCl_3$), (lit.[293] $[\alpha]_D^{24}$ +47.8° (*c* 0.96, $CHCl_3$)); IR ($CHCl_3$ cast) 3375 (O-H), 2930, 2865 (C-H), 1100, 1080, 755 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 3.65 (AB, 10 Hz, d, 3 Hz, 1H, CHOH), 3.40 (AB, 10 Hz, d, 6.5 Hz, 1H, CHOH), 3.36 (s, 3H, CH_3O), 2.79 (dd, 2.5, 2.5 Hz, 1H, CHOMe), 0.77-2.06 (m, 20H, CH, CH_2), 1.08 (d, obscure, 3H, CH_3-21), 1.04 (s, 3H, CH_3-19), 0.77 (s, 3H, CH_3-18), 0.66 (dd, 5.1, 3.6 Hz, 1H, 4-H), 0.44 (dd, 7.9, 5.0 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 82.21 (CHOH), 67.34 (CH_2OH), 56.21, 56.15, 52.60, 47.98, 43.15, 42.69, 40.04, 38.68, 35.03, 34.94, 33.27, 30.39, 27.61, 24.79, 24.14, 22.63, 21.20, 19.07, 16.64, 12.98, 12.14; exact mass: 346.2867 (346.2871 calcd for $C_{23}H_{38}O_2$).

Work-up of the ozonolysis mixture with dimethyl sulfide or zinc dust/AcOH[282] afforded a mixture of aldehydes **42** and **47** after chromatography (EtOAc 4%/hexane 96%); spectra were as for **42** (see below), except for ^1H NMR (400 MHz, $CDCl_3$) δ 9.582 (d, 3.3 Hz, CHO of **42**), 9.593 (d, 3.0 Hz, CHO of **47**).

6β-Methoxy-3α,5-cyclo-5α-pregnane-(20*S*)-20-carboxaldehyde (42)[290, 291]

The method of Corey and Suggs[294] was used. A solution of **46** (75.0 mg, 217 μmol) in CH_2Cl_2 (2 mL) was added to a stirred suspension of pyridinium chlorochromate (50.0 mg, 232 μmol) in CH_2Cl_2 (3 mL). The mixture was stirred for 45 min, and Et_2O

(10 mL) was added. The mixture was filtered through celite, which was then washed (Et_2O, 50 mL). The filtrates were concentrated *in vacuo*; column chromatography ($CHCl_3$) afforded:

Fraction (1), 46.4 mg (62%) of **42** as a clear oil: $[\alpha]_D^{24}$ +43.1° (*c* 0.940, $CHCl_3$); IR ($CHCl_3$ cast) 2932, 2889 (C-H), 1726 (C=O), 1098 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 9.58 (d, 3.2 Hz, 1H, CHO), 3.36 (s, 3H, CH_3O), 2.79 (dd, 2.4, 2.4 Hz, 1H, CHOMe), 2.38 (dd, 10.0, 3.2 Hz, q, 6.8 Hz, 1H, CHCHO), 0.8-2.0 (m, 19H, CH, CH_2), 1.14 (d, 6.8 Hz, 3H, CH_3-21), 1.05 (s, 3H, CH_3-19), 0.78 (s, 3H, CH_3-18), 0.66 (dd, 4.8, 4.0 Hz, 1H, 4-H), 0.44 (dd, 8.0, 5.2 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.87 (C=O), 82.38 (CHOMe), 56.50, 55.87, 51.32, 49.44, 48.19, 43.42, 40.07, 35.26, 35.12, 33.40, 33.40, 30.56, 27.12, 24.97, 24.53, 22.70, 21.44, 19.29, 13.47, 13.17, 12.60; exact mass: 344.2722 (344. 2729 calcd for $C_{23}H_{36}O_2$). Anal. calcd for $C_{23}H_{36}O_2$: C, 80.18; H, 10.53. Found: C, 80.51; H, 10.27. All samples prepared in this way contained 3% of **47** which had spectra as above except: ^1H NMR (400 MHz, $CDCl_3$) δ 9.59 (d, 3.0 Hz, CHO); ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.52 (CHO).

Fraction (2), recovered **46** (33%).

Aldehyde 42[290, 291] by Moffatt oxidation of 46

The method of Pfitzner and Moffatt[394] was used. Compound **46** (52.9 mg, 153 μmol) and dicyclohexylcarbodiimide (92.9 mg, 522 μmol) were dissolved in benzene (2 mL) and DMSO (0.25 mL). The mixture was cooled to 0 °C, and 72 μL of a solution of phosphoric acid (102 mg) in DMSO (1 mL) (63.6 μmol H_3PO_4) was added. The mixture was allowed to warm to room temperature and was stirred for 6 h. EtOAc (10 mL) was added, and the precipitate was collected and washed (EtOAc, 10 mL). The filtrate was washed (H_2O, 3 X 20 mL), dried and concentrated *in vacuo*. Column chromatography (EtOAc 4%/hexane 96%, then EtOAc 10%/hexane 90%) afforded:

Fraction (1), 3.29 mg (5%) of **48** as a gum: IR ($CHCl_3$ cast) 2946, 2928, 2861,

2849 (C-H), 1469, 1453, 1098, 1081, 1065 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.64, 4.60 (AB, 11.2 Hz, 2H, OCH_2S), 3.47 (AB, 8.8 Hz, d, 3.0 Hz, 1H, $CHHOCH_2S$), 3.27 (AB, 8.8 Hz, d, 7.2 Hz, 1H, $CHHOCH_2S$), 3.34 (s, 3H, CH_3O), 2.77 (dd, 2.8, 2.8 Hz, 1H, CHOMe), 2.15 (s, 3H, CH_3S), 0.75-2.00 (m, 20H, CH, CH_2), 1.04 (d, 6.8 Hz, 3H, CH_3-21), 1.02 (s, 3H, CH_3-19), 0.75 (s, 3H, CH_3-18), 0.65 (dd, 4.4, 4.4 Hz, 1H, 4-H), 0.43 (dd, 8.0, 5.2 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 82.47 (CHOMe), 75.54 and 73.62 (CH_2O, SCH_2O), 56.57, 56.32, 53.18, 48.16, 43.40, 42.96, 40.17, 36.54, 35.34, 35.12, 33.48, 30.56, 27.97, 25.04, 24.32, 22.79, 21.56, 19.38, 17.64, 13.92, 13.12, 12.38; exact mass: 406.2901 (406.2905 calcd for $C_{25}H_{42}O_2S$, 35%), 328.2766 (328.2766 calcd for $C_{23}H_{36}O$, M^+ - $CH_3SCH_2O\cdot$, 27%).

Fraction (2), 9.76 mg (19%) of **42**.

Fraction (3), 28.90 mg (55%) of **46**.

3-Methyl-1-butyl *p*-toluenesulfonate (52)

The method of Place *et al* [299] was used. To a solution of 3-methyl-1-butanol (11.1 mL, 100 mmol) and pyridine (24 mL) in CH_2Cl_2 (500 mL) was added *p*-toluenesulfonyl chloride (20.9 g, 110 mmol) with cooling at 0 °C. The mixture was stirred for 48 h at room temperature. The solution was washed (2 X H_2O, 250 mL), dried and concentrated *in vacuo*. Et_2O (250 mL) was added and the mixture was filtered. The solid was washed (Et_2O, 2 X 100 mL) and the combined filtrates concentrated *in vacuo* to afford 21.6 g (89%) of **52** as a semi-solid: IR ($CHCl_3$ cast) 1360, 1190, 1180 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 7.80, 7.35 (AB, 8 Hz, 4H, ArH), 4.05 (t, 6 Hz, 2H, CH_2O), 2.4 (s, 3H, $ArCH_3$), 1.55 (m, 3H, CH, CH_2), 0.85 (d, 6 Hz, 6H, 2 X CH_3); MS (EI) m/e 242 (M^+, 0.7%).

3-Methyl-1-iodobutane (53)

The method of Place *et al* [299] was used. To magnesium (5 g, 205 mmol) in Et_2O (50 mL) was slowly added iodine (39.3 g, 155 mmol). After all the iodine was added, and spontaneous refluxing ceased, the mixture was stirred until the color due to iodine had disappeared (ca. 30 min). The solution was cannelated into a dry flask, and a suspension of **52** (18.8 g, 77.5 mmol) in Et_2O (100 mL) was added. The mixture was stirred for 1 h, then ice-water (300 mL) was added. The ether layer was collected, washed (5% sodium thiosulfate, 100 mL), dried and concentrated at 200 mm Hg and room temperature on a rotary evaporator to give 9.30 g (61%) of **53** as a pale yellow liquid: IR ($CHCl_3$ cast) 2960 (C-H), 1360, 1168 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 3.23 (t, 7 Hz, 2H, CH_2I), 1.75 (m, 2H, CH_2), 1.38 (m, 1H, CH), 0.90 (d, 6 Hz, 6H, CH_3); MS (EI) m/e 198 (M^+, 0.7%), 70 (M^+ - HI, 100%).

(3-Methylbutyl)triphenylphosphonium iodide (49)[285]

The method of Bergmann and Dusza[285] was used. Compound **53** (5.67 g, 28.6 mmol), triphenylphosphine (5 g, 19.1 mmol) and toluene (25 mL) were heated at reflux for 48 h. The precipitate formed on cooling was collected, washed (toluene, 2 X 10 mL), and recrystallised from EtOH/H_2O to afford 6.87 g (78%) of crystalline **49**: mp 160 - 162 °C, (lit.[285] mp 174 - 176 °C); IR ($CHCl_3$ cast) 1438, 995 cm^{-1}, (lit.[285] 1435, 998 cm^{-1}); ^1H NMR (400 MHz, $CDCl_3$) δ 7.83 (m, 15H, ArH), 3.55 (m, 2H, CH_2P), 2.00 (septuplet, 6.8 Hz, t, 6.8 Hz, 1H, $CHMe_2$), 1.54 (m, 2H, CH_2CH_2P), 0.99 (d, 6.8 Hz, 6H, 2 X CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 135.32 (d, $^4J_{(31P-13C)}$= 2.5 Hz, 3C, *para*-ArC), 133.74 (d, $J_{(31P-13C)}$= 10 Hz) and 130.71 (d, $J_{(31P-13C)}$= 12.5 Hz) (12C, *ortho*-ArC, *meta*-ArC), 118.17 (d, $^1J_{(31P-13C)}$= 86.2 Hz, 3C, *ipso*-ArC), 31.05 (d, $^3J_{(31P-13C)}$= 4.2 Hz, $CHMe_2$), 28.86 (d, $^2J_{(31P-13C)}$= 14.8 Hz, CH_2CH_2P), 22.24 (2C, 2 X CH_3), 21.67 (d, $^1J_{(31P-13C)}$= 50.8 Hz, CH_2P); ^{31}P NMR (80 MHz, $CDCl_3$) δ 24.58 (br m, $w_{1/2}$= 50 Hz, 297K) or 1.55 (s, ^1H-decoupled, 300K); MS (EI) m/e 333 ($RPPh_3^+$).

Anal. calcd for $C_{23}H_{26}PI$: C, 60.01; H, 5.69; I, 27.57. Found: C, 59.75; H, 5.75; I, 27.92.

(2*R*)-2,3-Dimethylbutan-1-ol (51)[288]

A modification of the ozonolysis procedure of Partridge *et al*[293] was used. A solution of ergosterol (10.0 g, 25.2 mmol) in CH_2Cl_2 (250 mL) and pyridine (5 mL) was ozonised at -78 °C (ca. 87 mmol O_3) until a blue color appeared. The reaction vessel was flushed with N_2, and a solution of Red-Al™ in toluene (21 mL, 150 mmol) was added. The mixture was stirred for 1 h at -78 °C, and allowed to warm to room temperature over 1 h. Sulfuric acid (2 N, 250 mL) was added with caution and cooling. The resulting mixture was steam distilled until no further organic products distilled. The distillate was separated, and the organic phase dried and concentrated at 20 °C and 200 mm Hg. The residue was distilled in a kugelrohr (150 °C, 760 mm Hg). A small amount of the distillate was purified by preparative GLC (carbowax column); the last peak eluted was collected to afford **51**: IR ($CHCl_3$ cast) 3622 (sh, H-bonded O-H), 3460 (br, O-H), 2960, 2925, 2870 (C-H), 1465, 1010 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.72 (AB, 10 Hz, d, 6 Hz, 1H, CHOH), 3.48 (AB, 11 Hz, d, 7 Hz, 1H, CHOH), 1.73 (m, 1H, CH), 1.52 (m, 1H, CH), 1.30 (br s, 1H, OH), 0.92 (d, 8 Hz, 3H, CH_3), 0.87 (d, 7 Hz, 3H, CH_3), 0.85 (d, 7 Hz, 3H, CH_3); GC-MS m/e 102 (M^+); $[\alpha]_D^{24}$ of the crude material was negative, cf. the (*S*)-enantiomer[288] which has positive rotation.

(2*R*)-2,3-Dimethylbutyl *p*-toluenesulfonate (54)

The method of Place *et al*[299] was modified. To a solution of **51** in CH_2Cl_2 (500 mL), prepared as above from ergosterol (50.2 g, 127 mmol), was added pyridine (44 g) and *p*-toluenesulfonyl chloride (106 g, 560 mmol). The mixture was stirred for 24 h, washed (H_2O, 2 X 50 mL), dried and concentrated *in vacuo*. Multiple column chromatography (EtOAc 4%/hexane 96%) gave 2.91 g (9.0% based on ergosterol) of **54**

as an oil: $[\alpha]_D^{24}$ -4.5° (*c* 5.45, $CHCl_3$); IR ($CHCl_3$ cast) 2960, 2875 (C-H), 1598, 1465, 1360, 1190, 1178, 965, 840, 815, 665 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.79, 7.35 (AB, 8.2 Hz, 4H, ArH), 3.94 (AB, 9.2 Hz, d, 5.6 Hz, 1H, CHOTs), 3.85 (AB, 9.2 Hz, d, 6.3 Hz, 1H, CHOTs), 2.44 (s, 3H, $ArCH_3$), 1.66 (m, 2H, CH-CH), 0.832 (d, 6.6 Hz, 3H, CH_3), 0.822 (d, 6.6 Hz, 3H, CH_3), 0.762 (d, 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.75 and 133.37 (wk, 2 X ArC), 129.90 and 127.93 (4C, ArCH), 73.86 (CH_2OTs), 38.44, 28.84, 21.59, 20.20, 17.99, 12.59; MS (NH_3-CI) m/e 274 ($M+NH_4^+$, 100%). Anal. calcd for $C_{13}H_{20}O_3S$: C, 60.91; H, 7.86. Found: C, 60.92; H, 7.75.

(2*R*)-2,3-Dimethyl-1-iodobutane (55)[288]

The procedure[299] for conversion of **52** to **53** was used. Thus, **54** (610 mg, 2.38 mmol) afforded 280 mg (56%) of crude **55** as a very unstable liquid: IR ($CHCl_3$ cast) 2960, 2945, 2855 (C-H), 1490, 805 cm^{-1}; ^1H NMR (80 MHz, $CDCl_3$) δ 3.22 (m, 2H, CH_2I), 1.75 (m, 2H, CH-CH), 0.82-1.00 (3 X d, 9H, 3 X CH_3).

((2*R*)-2,3-Dimethyl-1-butyl)triphenylphosphonium iodide (50)[288]

The method of Bergmann and Dusza[285] was used. A mixture of **55** (280 mg, 1.3 mmol), triphenylphosphine (1.00 g, 3.82 mmol) and toluene (5 mL) was heated at reflux for 3 days in the dark. The mixture was cooled, and the solvent was decanted from the separated oil, which was washed (toluene, 2 X 10 mL). The oil was redissolved in boiling toluene (5 mL, 2 h) and the mixture was cooled. Excess solvent was again decanted leaving 210 mg (34%) of **50** as a gum: $[\alpha]_D^{24}$ -8.9° (*c* 3.20, $CHCl_3$); IR ($CHCl_3$ cast) 3050, 3005 (Ar-H), 2960, 2870 (C-H), 1437, 1112, 995, 746, 721, 690 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.85 (m, 15H, ArH), 3.48 (AB, 16.0 Hz, dd, 12.4, 8.2 Hz, 1H, CHP), 3.40 (AB, 15.2 Hz, dd, 14.0, 3.8 Hz, 1H, CHP), 1.88 (m, 1H, CH), 1.75 (m, 1H, CH), 0.919 (d, 6.8 Hz, 3H, CH_3), 0.867 (d, 6.8 Hz, 3H, CH_3), 0.816 (d, 6.8 Hz, 3H, CH_3);

^{13}C NMR (100 MHz, $CDCl_3$) δ 134.34 (3C, *para* -ArC), 132.76 (d, $J_{(31P-13C)}$= 10 Hz) and 129.78 (d, $J_{(31P-13C)}$= 12 Hz) (12C, *ortho* -ArC, *meta* -ArC), 117.59 (wk, d, $^1J_{(31P-13C)}$= 86 Hz, 3C, *ipso* -ArC), 33.07, 32.61 (d, $^2J_{(31P-13C)}$= 11 Hz, $CHCH_2P$), 27.15 (d, $^1J_{(31P-13C)}$= 49 Hz, CHP), 19.29, 16.40, 15.36 (d, $^3J_{(31P-13C)}$= 3.9 Hz). MS (EI) m/e 277 ($CH_2=PPh_3^+$, 100%).

(22*E*)-6β-Methoxy-3α,5-cyclo-5α-cholest-22-ene (56) and (22*Z*)-6β-methoxy-3α,5-cyclo-5α-cholest-22-ene (57) by the Wittig reaction[282-285]

Literature methods[282-285] were adapted. To a stirred suspension of **49** (367 mg, 797 μmol) in THF (5 mL) was added n-butyllithium (1.55 M in hexane, 0.51 mL, 791 μmol). The mixture was stirred for 15 min, and the orange solution cooled to -78 °C. A solution of **42** (279 mg, 810 μmol) in THF (2 mL) was added dropwise. The mixture was stirred for 30 min at -78 °C, and allowed to warm to room temperature. After stirring for 30 min, the mixture was heated at reflux for 30 min, then MeOH (1 mL) was added. After a further 30 min at reflux, the solution was concentrated *in vacuo*. Column chromatography ($CHCl_3$) afforded a tan oil. Repeated column chromatography (EtOAc 4%/hexane 96%) gave 150 mg (47%) of a mixture of **56** and **57**, in a ratio of 12:88 determined by comparison of the intensities of the ^{13}C NMR signals at δ 137.29 and 125.43 for **57**, and at 138.19 and 126.13 for **56** (see below for full spectra).

Wittig reactions in hexane[282] (8 mL) or Et_2O (3 mL) were performed similarly, except that **42** was added at reflux for hexane, or at room temperature for Et_2O. The yields of the purified mixture of **56** and **57** were 10% in hexane and 32% in Et_2O.

6β-Methoxy-3α,5-cyclo-5α-cholest-22-enes 56 and 57 by the Wittig-Schlosser reaction[302, 303]

The procedure of Johnson *et al*[395] was adapted. A solution of n-butyllithium (1.42 M in hexane, 16 mL, 22.7 mmol) was added to a stirred suspension of **49** (10.2 g, 22.3

mmol) in Et_2O (200 mL). The mixture was stirred for 20 min, then cooled to -78 °C. A solution of **42** (7.27 g, 21.1 mmol) in Et_2O (120 mL) was added, and the mixture was stirred for 5 min. n-Butyllithium (1.42 M in hexane, 16 mL, 22.7 mmol) was added, and the mixture was stirred at -78 °C for 1 h, then warmed to -25 °C and stirred for 15 min. MeOH (ca. 0.8 mL) was added dropwise until the orange colour nearly disappeared, and the mixture was warmed slowly to room temperature, then heated at reflux for 90 min. Hexane (300 mL) was added, and the mixture heated at reflux for 20 h. MeOH (20 mL) was added, and the mixture was heated at reflux for 30 min, then concentrated *in vacuo*. Column chromatography ($CHCl_3$) gave:

Fraction (1), an oil. Further column chromatography (EtOAc 4%/hexane 96%) afforded 2.35 g (28%) of a mixture of **56** and **57**. The ratio of **56** to **57** was determined (as above) to be 75:25.

Fraction (2), 3.62 g (42%) of (22χ)-22-hydroxy-6β-methoxy-3α,5-cyclo-26-norcholestane **95** as an oil: $[\alpha]_D^{24}$ +32.2° (*c* 0.326, $CHCl_3$); IR ($CHCl_3$ cast) 3430 (O-H), 2952, 2933, 2868 (C-H), 1467, 1459, 1381, 1099, 1085 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.65 (dd, 7.8, 4.0 Hz, 1H, C<u>H</u>OH), 3.33 (s, 3H, CH_3O), 2.76 (dd, 2.8, 2.8 Hz, 1H, C<u>H</u>OMe), 0.85-2.00 (m, 27H, C<u>H</u>, CH_2), 0.976 (s, 3H, CH_3-19), 0.860 (t, 6.4 Hz, 3H, CH_3CH_2), 0.844 (d, 6.4 Hz, 3H, CH_3-21), 0.680 (s, 3H, CH_3-18), 0.60 (dd, 4.4, 4.4 Hz, 1H, 4-H), 0.38 (dd, 8.0, 5.2 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 81.53 (<u>C</u>HOMe), 72.64 (<u>C</u>HOH), 55.64, 55.57, 51.89, 47.12, 42.45, 41.70, 39.46, 39.36, 34.38, 34.20, 34.16, 32.45, 29.69, 27.84, 26.92, 24.06, 23.20, 21.87, 21.87, 20.52, 18.33, 13.18, 12.10, 11.26, 10.51; exact mass: 402.3497 (402.3497 calcd for $C_{27}H_{46}O_2$).

Fraction (3), 0.733 g (9%) of (22χ)-22-hydroxy-6β-methoxy-3α,5-cyclo-26-norcholestane **96** as an oil: $[\alpha]_D^{24}$ +21.2° (*c* 1.87, $CHCl_3$); IR ($CHCl_3$ cast) 3402 (br, O-H), 2961, 2928, 2859 (C-H), 1467, 1457, 1377, 1098 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.64 (m, 1H, C<u>H</u>OH), 3.36 (s, 3H, CH_3O), 2.75 (dd, 2.8, 2.4 Hz, 1H,

CHOMe), 1.96 (d, ≈13 Hz, m, 1H, CH, CH_2), 1.87 (d, ≈13 Hz, m, 1H, CH, CH_2), 0.75-1.80 (m, 28H, CH, CH_2, CH_3CH_2), 1.02 (s, 3H, CH_3-19), 0.90 (d, 6.7 Hz, 3H, CH_3-21), 0.73 (s, 3H, CH_3-18), 0.62 (dd, 4.8, 3.6 Hz, 1H, 4-H), 0.41 (dd, 8.0, 5.0 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 82.46 (CHOMe), 73.40 (CHOH), 56.07, 55.72, 52.81, 47.60, 42.57, 42.24, 41.59, 39.46, 34.32, 34.17, 32.55, 29.95, 29.03, 28.38, 26.36, 24.35, 23.62, 22.18, 21.87, 18.67, 13.24, 12.23, 11.51, 11.42; exact mass: 402.3503 (402.3497 calcd for $C_{27}H_{46}O_2$).

Multiple column chromatography of the mixture of **56** and **57** (alumina impregnated with 25% w/w silver nitrate, Et_2O 1%/benzene 13%/hexane 86%)[283] afforded:

Fraction (1), 1.51 g (18%) of **56**, which contained less than 2% of **57**, as a white solid: mp 60.5 - 61 °C; $[\alpha]_D^{24}$ +33.1° (*c* 2.40, $CHCl_3$); IR ($CHCl_3$ cast) 2953, 2932, 2868 (C-H), 1455 (br), 1099, 965 (*trans* -C=C) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.28 (AB, 15.0 Hz, dd, 6.6, 6.6 Hz, 1H, CH=CHCH$_2$), 5.24 (AB, 15.2 Hz, d, 8.0 Hz, 1H, CH=CHCH$_2$), 3.34 (s, 3H, CH_3O), 2.76 (dd, 2.4, 2.4 Hz, 1H, CHOMe), 0.7-2.1 (m, 23H, CH, CH_2), 1.025 (s, 3H, CH_3-19), 1.003 (d, 6.6 Hz, 3H, CH_3-21), 0.861 (d, 6.8 Hz, 3H, CH_3-26), 0.857 (d, 6.8 Hz, 3H, CH_3-27), 0.731 (s, 3H, CH_3-18), 0.64 (dd, 5.0, 2.0 Hz, 1H, 4-H), 0.42 (dd, 8.0, 5.6 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.19 and 126.13 (CH=CH), 82.45 (CHOMe), 56.68, 56.53, 56.17, 48.16, 43.42, 42.71, 42.02, 40.19, 35.32, 35.14, 33.40, 30.51, 28.75, 28.57, 24.99, 24.21, 22.80, 22.35, 22.30, 21.48, 20.89, 19.30, 13.12, 12.46; exact mass: 398.3548 (398.3548 calcd for $C_{28}H_{46}O$).

Fraction (2), 290 mg (3%) of a mixture of **56** and **57**.

Fraction (3), 0.363 g (4%) of **57** as a clear oil: $[\alpha]_D^{24}$ +14.5° (*c* 1.17, $CHCl_3$); IR ($CHCl_3$ cast) 2953, 2932, 2868 (C-H), 1455, 1099 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.19 (m, fit for AXBX'X", J_{AB}=10.8 Hz, J_{AX}=7.4 Hz, $J_{BX'}$= $J_{BX"}$= 6.4 Hz, 2H, CH=CH), 3.32 (s, 3H, CH_3O), 2.76 (dd, 2.7, 2.7 Hz, 1H, CHOMe), 2.43 (m,

1H, C<u>H</u>, C<u>H</u>$_2$), 0.7-2.0 (m, 22H, C<u>H</u>, C<u>H</u>$_2$), 1.030 (s, 3H, C<u>H</u>$_3$-19), 0.950 (d, 6.0 Hz, 3H, C<u>H</u>$_3$-21), 0.905 (d, 6.0 Hz, 3H, C<u>H</u>$_3$-26), 0.890 (d, 6.6 Hz, 3H, C<u>H</u>$_3$-27), 0.756 (s, 3H, C<u>H</u>$_3$-18), 0.66 (dd, 4.4, 4.4 Hz, 1H, 4-<u>H</u>), 0.43 (dd, 8.0, 5.2 Hz, 1H, 4-<u>H</u>); ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.29 and 125.47 (<u>C</u>H=<u>C</u>H), 82.43 (<u>C</u>HOMe), 56.66, 56.56, 56.47, 48.18, 43.44, 42.74, 40.29, 36.81, 35.35, 35.08, 34.26, 33.41, 30.52, 28.76, 28.19, 24.99, 24.19, 22.81, 22.63, 22.39, 21.54, 20.71, 19.33, 13.10, 12.58; exact mass: 398.3556 (398.3548 calcd for $C_{28}H_{46}O$). Anal. calcd for $C_{28}H_{46}O$: C, 84.36; H, 11.63. Found: C, 83.99; H, 11.32.

Fractions from the argentation chromatography column were analysed either by GC (OV-101 column, 230 °C, retention times: **56**, 42.1 min; **57**, 39.9 min) or more conveniently by ^{13}C NMR.

(20*S*)-20-*p*-Toluenesulfonoxymethyl-6β-methoxy-3α,5-cyclo-5α-pregnane (59)[293]

The method of Partridge *et al*[293] was used. A solution of *p*-toluenesulfonyl chloride (330 mg, 1.73 mmol) in pyridine (450 μL) was added dropwise to a solution of **46** (427 mg, 1.23 mmol) in pyridine (550 μL) at 0 °C. The mixture was stirred for 3 h at 0 °C. Ice (10 g) was added, and the mixture was extracted (CH_2Cl_2, 2X 15 mL). The extracts were dried and concentrated *in vacuo* to give 435 mg (71%) of crude **59** as a yellow solid. Chromatography resulted in decomposition. mp 120 - 122 °C, (lit.[293] mp 144 - 145 °C); $[\alpha]_D^{24}$ +35.6° (*c* 0.975, $CHCl_3$), (lit.[293] $[\alpha]_D^{24}$ +30.8° (*c* 1.00, $CHCl_3$)); IR ($CHCl_3$ cast) 3060 (Ar-H), 2933, 2868 (C-H), 1361, 1188, 1177, 1097, 952 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73, 7.30 (AB, 8 Hz, 4H, Ar<u>H</u>), 3.98 (AB, 10 Hz, d, 3 Hz, 1H, C<u>H</u>HOTs), 3.80 (AB, 10 Hz, d, 7 Hz, 1H, CH<u>H</u>OTs), 3.34 (s, 3H, C<u>H</u>$_3$O), 2.78 (br s, 1H, C<u>H</u>OMe), 2.46 (s, 3H, ArC<u>H</u>$_3$), 1.05-2.00 (m, 17H, C<u>H</u>, C<u>H</u>$_2$), 1.03 (s, 3H, C<u>H</u>$_3$-19), 0.99 (d, 7 Hz, 3H, C<u>H</u>$_3$-21), 0.68 (s, 3H, C<u>H</u>$_3$-18), 0.83 (m, 3H, C<u>H</u>, C<u>H</u>$_2$), 0.64 (dd, 5, 4 Hz, 1H, 4-<u>H</u>), 0.43 (dd, 8, 5 Hz, 1H, 4-<u>H</u>); ^{13}C NMR

(100 MHz, $CDCl_3$) δ 144.52 and 133.18 (wk, ArC), 129.71 and 127.84 (4C, ArCH), 82.25 (CHOMe), 75.60 (CH_2OTs), 56.47, 56.03, 51.87, 47.84, 43.27, 42.79, 39.83, 38.69, 36.11, 35.13, 34.98, 33.26, 30.42, 27.70, 27.42, 24.86, 24.05, 22.59, 21.53, 21.36, 19.16, 12.99, 12.10; exact mass: 500.2954 (500.2961 calcd for $C_{30}H_{44}SO_4$).

3β-Iodo-(20*S*)-20-(iodomethyl)-pregn-5-ene (61)

A modification of the procedure of Place *et al* [299] was used. Magnesium iodide (15.4 mmol) in Et_2O was prepared as for the conversion of **52** into **53**, and added to a suspension of **59** (349 mg, 697 μmol) in Et_2O (5 mL). The mixture was stirred for 1 h, and poured into H_2O (20 mL). One crystal of sodium bisulfite was added to remove iodine, and the solution was extracted (Et_2O, 2 X 20 mL). The extracts were dried and concentrated *in vacuo*. Recrystallisation from hexane afforded 238 mg (62%) of **61** as white crystals: mp 129 - 130 °C dec. (decomposes slowly above 120 °C); $[\alpha]_D^{24}$ -6.7° (*c* 1.98, $CHCl_3$); IR ($CHCl_3$ cast) 2940, 2900, 2870 (C-H), 1462, 1435, 1380 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.34 (ddd, 5.0, 2.0, 2.0 Hz, 1H, CH=C), 4.03 (dddd, 12.4, 12.4, 4.4, 4.4 Hz, 1H, 3α-H), 3.33 (AB, 10.0 Hz, d, 1.8 Hz, 1H, CHHI), 3.17 (AB, 9.6 Hz, d, 4.8 Hz, 1H, CHHI), 2.93 (AB, 13.2 Hz, dd, 2.0, 2.0 Hz, 1H, 4α-H), 2.67 (AB, 13.6 Hz, dd, 4.2, 2.0 Hz, 1H, 4β-H), 2.26 (m, 2H, CH, CH_2), 0.9-2.0 (m, 17H, CH, CH_2), 1.05 (s, 3H, CH_3-19), 1.03 (d, 5.6 Hz, 3H, CH_3-21), 0.72 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 142.87 (wk, C=CH), 121.52 (C=CH), 56.32, 55.48, 50.27, 46.41, 42.39, 41.90, 39.46, 36.91, 36.67, 36.40, 31.72, 31.68, 30.13, 27.52, 24.18, 20.96, 20.85, 20.78, 19.23, 12.60; MS (isobutane-CI) m/e 553 (MH^+, 1%), 425 (MH^+ - HI, 59%), 296 (M^+ - 2HI, 100%). Anal. calcd for $C_{22}H_{34}I_2$: C, 47.84; H, 6.20; I, 45.95. Found: C, 48.00; H, 6.20; I, 45.78.

(20*S*)-20-Iodomethyl-6β-methoxy-3α,5-cyclo-5α-pregnane (60)[292, 293]

The procedure of Partridge *et al* [293] was followed. A solution of **59** (399 mg, 798

μmol) and sodium iodide (1.22 g, 8.13 mmol) in acetone (16 mL) was stirred at reflux for 16 h. The mixture was poured into 10% sodium bisulfite (100 mL) and the solution was extracted (EtOAc, 2 X 50 mL). The extracts were washed (brine, 20 mL), dried and concentrated *in vacuo*. Column chromatography ($CHCl_3$) afforded 231 mg (64%) of **60** as a white solid: mp 100 - 101 °C, (lit.[293] mp 103 - 104 °C); $[\alpha]_D^{24}$ +56.1° (*c* 1.085, $CHCl_3$), (lit.[293] +56.7° (*c* 1.09, $CHCl_3$)); IR ($CHCl_3$ cast) 2930, 2865 (C-H), 1098 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.33 (AB, obscure, d, 1.6 Hz, 1H, CHHI), 3.32 (s, 3H, CH_3O), 3.17 (AB, 9.2 Hz, d, 4.4 Hz, 1H, CHHI), 2.76 (dd, 2.6, 2.6 Hz, 1H, CHOMe), 0.7-2.0 (m, 20H, CH, CH_2), 1.019 (s, 3H, CH_3-19), 1.019 (d, 4.5 Hz, 3H, CH_3-21), 0.731 (s, 3H, CH_3-18), 0.62 (dd, 5.0, 4.0 Hz, 1H, 4-H), 0.41 (dd, 8.0, 5.6 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 82.31 (CHOMe), 56.52, 56.20, 55.58, 47.86, 43.31, 42.89, 40.07, 36.95, 35.23, 35.01, 33.47, 30.55, 27.60, 24.92, 24.08, 22.77, 21.41, 21.02, 20.78, 19.23, 13.11, 13.02; exact mass: 456.1904 (456.1889 calcd for $C_{23}H_{37}OI$). Anal. calcd for $C_{23}H_{37}OI$: C, 60.52; H, 8.17; I, 27.80. Found: C, 60.82; H, 8.17; I, 27.85.

(20*S*)-20-[6β-Methoxy-3α,5-cyclo-5α-pregnanyl]-methyl-triphenylphosphonium iodide (58)[289]

The procedure of Barner *et al*[289] was used. A mixture of **60** (92.0 mg, 202 μmol), triphenylphosphine (66.9 mg, 255 μmol) and potassium carbonate (66.2 mg) in CH_3CN (5 mL) was heated at reflux for 2 weeks. The mixture was cooled, diluted with acetone (10 mL) and filtered through celite (5 g), which then was washed with acetone (2 X 30 mL). The filtrates were concentrated *in vacuo*. The residue was triturated with Et_2O (10 mL) to afford **58** as a gum: $[\alpha]_D^{24}$ +40.0° (*c* 1.24, acetone); IR ($CHCl_3$ cast) 3050 (Ar-H), 2931, 2867 (C-H), 1438, 1111, 1100, 955 (wk), 750, 691 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.3-8.0 (m, 15H, ArH), 4.02 (dd, 16, 11 Hz, d, $^2J_{(^1H-^{31}P)}$= 11 Hz, 1H, CHHP), 3.30 (s, 3H, CH_3O), 3.12 (d, 16 Hz, d, $^2J_{(^1H-^{31}P)}$= 15 Hz, 1H, CHHP),

2.77 (dd, 2, 2 Hz, 1H, CHOMe), 0.7-2.3 (m, 19H, CH, CH_2), 1.00 (d, 5.6 Hz, 3H, CH_3-21), 0.98 (s, 3H, CH_3-19), 0.64 (m, 2H, 3-H, 4-H), 0.53 (s, 3H, CH_3-18), 0.43 (dd, 6.4, 4.8 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 134.66 (3C, *para*-ArC), 133.19 (d, $J_{(^{13}C-^{31}P)}$= 10 Hz) and 130.15 (d, $J_{(^{13}C-^{31}P)}$= 12 Hz) (12C, *ortho*-ArC, *meta*-ArC), 118.32 (wk, d, $^1J_{(^{13}C-^{31}P)}$= 85 Hz, *ipso*-ArC), 81.75 (CHOMe), 56.00, 55.79 (d, $J_{(^{13}C-^{31}P)}$= 13 Hz), 55.49, 47.08, 42.68 (d, $J_{(^{13}C-^{31}P)}$= 14 Hz), 39.25, 34.71, 34.29, 32.82, 32.45, 32.41, 29.93, 28.81 (d, $^1J_{(^{13}C-^{31}P)}$= 47 Hz, CH_2P), 28.40, 24.39, 23.38, 22.12, 20.94, 20.21, 18.68, 12.53, 11.48.

6β-Methoxy-3α,5-cyclo-5α-cholest-22-enes 56 and 57 from 58 by the Wittig-Schlosser reaction

The method for preparation of **56** and **57** from **42** was used. Thus, **58** (80.0 mg, 111μmol) and 3-methylbutanal condensed to afford 22.1 mg (50%) of a mixture of **56** and **57**; ^{13}C NMR analysis revealed a ratio for **56**:**57** of 29:71.

(22*E*)-Cholesta-5,22-dien-3β-ol (9)[281-283]

The method[267] for conversion of **35** to **8** was used. Thus, **56** (47.1 mg, 118 μmol) afforded 43.1 mg (95%) of pure **9** as a white solid which was recrystallised (acetone): mp 129 - 130 °C, (lit.[281] mp 133.5 - 134 °C); $[\alpha]_D^{24}$ -54.4° (*c* 0.97, $CHCl_3$), (lit.[281] $[\alpha]_D^{24}$ -57.3° (*c* 1.22, $CHCl_3$)); IR ($CHCl_3$ cast) 3360 (br, O-H), 2953, 2934, 2899, 2867 (C-H), 1463, 1365, 1059, 970, 960 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.35 (br d, 5 Hz, 1H, CH=C), 5.26 (AB, 15.0 Hz, dd, 6.5, 6.5 Hz, 1H, 23-HC=CH), 5.23 (AB, 15.0 Hz, d, 7.5 Hz, 1H, 22-HC=CH), 3.53 (dddd, 10.4, 10.4, 4.8, 4.8 Hz, 1H, CHOH), 2.30 (m, 2H, CH, CH_2), 0.9-2.1 (m, 23H, CH, CH_2),1.100 (d, 6.5 Hz, 3H, CH_3-21), 1.100 (s, 3H, CH_3-19), 0.865 (d, 6.5 Hz, 3H, CH_3-26), 0.858 (d, 6.5 Hz, 3H, CH_3-27), 0.690 (s, 3H, CH_3-18); ^{13}C NMR (75 MHz, $CDCl_3$) δ 140.77 (wk, C=CH), 138.13, 126.25 and 121.72 (CH=C, CH=CH), 71.83 (CHOH), 56.87,

55.95, 50.18, 42.32, 42.24, 41.97, 40.12, 39.69, 37.27, 36.52, 31.92, 31.92, 31.68, 28.64, 28.56, 24.30, 22.31, 22.26, 21.08, 20.85, 19.40, 12.06; exact mass: 384.3385 (384.3378 calcd for $C_{27}H_{44}O$). Anal. calcd for $C_{27}H_{44}O$: C, 84.38; H, 11.46. Found: C, 83.99; H, 11.32.

(22*RS*, 23*SR*)-3β-Acetoxyergosta-5,7-diene-22,23-epoxide N-Phenyltriazoline-3,5-dione Adducts (62)[308]

The method of Crump *et al*[308] was used. A mixture of **31** (3.14 g, 5.12 mmol), *meta* -chloroperoxybenzoic acid (1.23 g, 7.13 mmol) and CH_2Cl_2 (40 mL) was stirred at room temperature for 20 h. The solution was filtered through alumina (Woelm neutral grade IV, 10 g) which was washed (CH_2Cl_2, 100 mL). The filtrates were concentrated *in vacuo* to afford 3.19 g (99%) of **62** as a foam: IR ($CHCl_3$ cast) 2960, 2870 (C-H), 1755 (C=O amide), 1735 (C=O ester), 1703 (C=O amide), 1397, 1242, 753 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.42, 7.30 (m, 5H, ArH), 6.40, 6.27 (AB, 8.0 Hz, CH=CH), 5.47 (m, 1H, CHOAc), 3.23 (dd, 16.0, 4.0 Hz, 1H, 4α-H), 2.70-2.00 (m, 6H, 22-H, 23-H, CH, CH_2), 2.01 (s, 3H, CH_3CO_2), 1.20-1.90 (m, 13H, CH, CH_2), 0.88-1.12 (sh m, 18H, 6 X CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) showed 52 peaks, consistent with a mixture of stereoisomers; MS (NH_3-CI) m/e 647 ($[M + NH_3]^+$, 3%), 394 (M^+ - AcOH - **24**, 100%).

(22*RS*, 23*SR*)-6,7-Dihydro-(3β-acetoxyergosta-5,7-diene-22,23-epoxide N-phenyltriazoline-3,5-dione adducts) (63), and N-cyclohexyl analogues (64)

The method[256] for the conversion of **26** to **27** was used. Thus, **62** (25.7 mg, 40.9 μmol) afforded 24.0 mg (93%) of a mixture of **63** and **64** as a clear oil: IR ($CHCl_3$ cast) 2955, 2865 (C-H), 1748 (C=O amide), 1730 (C=O ester), 1695 (C=O amide) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.48 (m, ArH), 5.63 (dddd, 10, 10, 5, 5 Hz, 1H,

CHOAc), 2.81 (dd, 14.0, 5.0 Hz, 1H, 4α-H), 2.68 (dd, 8.0, 1.8 Hz, 1H, CH (epoxide)), 2.59 (dd, 6.0, 2.2 Hz, 1H, CH (epoxide)), 2.01 (s, 3H, CH_3CO_2), 1.08-2.54 (m, 23H, CH, CH_2), 1.142 and 1.132 (2 X s, 3H, CH_3-19 for each diastereomer), 0.87-1.02 (sh m, 12H, 4 X CH_3), 0.845 (s, 3H, CH_3-18); exact mass: 631.4054 (631.3986 calcd for $C_{38}H_{53}N_3O_5$, **63**$^+$, 45%), 637 (**64**$^+$, 13%).

(22χ,23χ)-Ergosta-5,7-diene-3β,22,23-triol N-phenyltriazoline-3,5-dione adducts (66) and (22*RS*, 23*SR*)-ergosta-5,7-dien-3β-ol-22,23-epoxide N-phenyltriazoline-3,5-dione adducts (65)[393]

A modification of the procedure of Winstein and Henderson[393] was used. **62** (1.00 g, 1.59 mmol) was dissolved in THF (30 mL) and 50% H_2SO_4 (10 mL) was added. The mixture was stirred for 7 days, and 10% Na_2CO_3 was added until the mixture was basic. THF was removed on the rotary evaporator, and the residue was extracted ($CHCl_3$, 3 X 20 mL). The extracts were dried and concentrated *in vacuo* . Column chromatography (MeOH 5%/$CHCl_3$ 95%) gave:

Fraction (1), 252 mg (27%) of **65** as a white solid: IR ($CHCl_3$ cast) 3440, 3340 (br, O-H), 2960, 2875 (C-H), 1750, 1700 (C=O amide), 1400, 757 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.44, 7.30 (m, 5H, ArH), 6.37, 6.27 (2 X AB, 8 Hz, 2H, CH=CH), 4.42 (m, 1H, CHOH), 3.17 (dd, 14, 4 Hz, 1H, 4α-H), 2.2-3.0 (m, 5H, 22-CH, 23-CH, CH, CH_2), 1.2-2.2 (m, 19H, CH, CH_2), 0.8-1.2 (sh m, 18H, 6 X CH_3); UV (MeOH) λ_{max} (logε) 270 (3.48); MS (NH_3-CI) m/e 604 ([M + NH_3]$^+$, 2%), 393 (M$^+$ - H_2O - **24**, 21%); ^{13}C NMR (100 MHz, $CDCl_3$) shows a preponderance of one isomer.

Fraction (2), 290 mg (30%) of **66** as a white solid: mp 159 - 160 °C; $[\alpha]_D^{24}$ - 49.2° (*c* 0.98, MeOH); IR (MeOH cast) 3470 (str, O-H), 2950, 2870 (C-H), 1750, 1699 (C=O amide), 1398, 755 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$ 50%/CD_3OD 50%) δ 7.38 (m, 5H, ArH), 6.55, 6.35 (AB, 8.4 Hz, 2H, CH=CH), 4.32 (m, 1H, CHOH), 3.66, 3.54 (AB, 10 Hz, m, =2 Hz, 2H, 22-H, 23-H), 3.09 (dd, 14, 5 Hz, 1H, 4α-H), 2.37 (m, 2H,

CH, CH_2), 1.08-2.20 (m, 20H, CH, CH_2), 1.009 (s, 3H, CH_3-19), 0.960 (d, 6.4 Hz, 3H, CH_3), 0.950 (2 X d, 6.4 Hz, 6H, 2 X CH_3), 0.901 (s, 3H, CH_3-18), 0.848 (d, 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 149.95 and 147.37 (wk, 2 X C=O amide), 137.29, 130.09 and 129.30 (CH=CH, *para*-ArC), 133.09 (wk, *ipso*-ArC), 130.03 and 128.03 (4C, *ortho*-ArC, *meta*-ArC), 73.31, 72.27 and 67.78 (3-C, 22-C, 23-C), 67.27 and 66.61 (wk, 5-C, 8-C), 54.28, 53.33, 50.71, 45.12, 42.34, 40.96, 39.83, 37.12, 35.24, 35.10, 32.14, 30.38, 27.91, 24.11, 23.42, 21.57, 21.19, 18.02, 13.34, 12.60, 10.05; UV λ_{max} (logε) 220 (infl., 4.09), 254 (3.63) nm; MS (NH_3-CI) m/e 623 ($[M + NH_3]^+$, 0.1%), 446 ($[M + NH_3]^+$ - **24**, 18%), 428 (M^+ - **24**, 10%), 410 (M^+ - H_2O - **24**, 100%), 392 (M^+ - $2H_2O$ - **24**, 5%).

Replacement of H_2SO_4 with 35% $HClO_4$ also afforded **66** but in lower yield; a correspondingly larger amount of **65** was isolated. When **62** (216 mg, 343 μmol) was heated at reflux with periodic acid (298 mg, 1.55 mmol) in acetone (20 mL) and H_2O (3 mL) for 48 h, work-up as above gave 194 mg (96%) of **65**.

(22*RS*, 23*SR*)-6,7-Dihydro-(ergosta-5,7-dien-3β-ol-22,23-epoxide N-phenyltriazoline-3,5-dione adducts) (67) and N-cyclohexyl analogues (69)

The method[256] used to convert **26** to **27** was used. Thus, **65** (22.5 mg, 38.2 μmol) gave 17.9 mg (79%) of a mixture of **67** and **69** as an oil: IR ($CHCl_3$ cast) 3440 (O-H), 2958, 2934, 2872 (C-H), 1747, 1691 (C=O amide), 1406, 753 cm^{-1}; exact mass: 595.4322 (595.4349 calcd for $C_{36}H_{57}N_3O_4$, **69**$^+$, 4%), 589.3882 (589.3880 calcd for $C_{36}H_{51}N_3O_4$, **67**$^+$, 15%).

(22χ,23$\hat{\chi}$)-6,7-Dihydro-(ergosta-5,7-diene-3β,22,23-triol N-phenyltriazoline-3,5-dione adduct) (68)

The method[256] used to convert **26** to **27** was used. Thus, **66** (53.7 mg, 88.6 μmol) afforded 27.2 mg (51%) of **68** as a white solid: mp 152 - 153 °C (becomes

gummy), 200 - 202 °C (melts with decomposition); $[\alpha]_D^{24}$ -55.4° (*c* 0.500, MeOH); IR ($CHCl_3$ cast) 3450 (O-H), 2960, 2870 (C-H), 1745, 1688 (C=O amide), 1409, 753 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.46 (m, 5H, ArH), 4.56 (m, 1H, 3-CHOH), 3.68, 3.54 (AB, 8 Hz, m, CHOH-CHOH), 2.73 (dd, 13.5, 4.5 Hz, 1H, 4α-H), 1.0-2.5 (m, 24H, CH, CH_2), 1.13 (s, 3H, CH_3), 0.969, 0.949, 0.937, 0.920, 0.875, 0.868, 0.852, 0.834 (sharp, 15H, 5 X CH_3); ^{13}C NMR (90 MHz, $CDCl_3$) δ 146.91 and 145.12 (2 X C=O), 132.08 (*ipso*-ArC), 128.89 and 126.53 (4C, *ortho*-ArCH, *meta*-ArCH), 127.98 (*para*-ArCH), 72.67, 72.51, 67.60 (3 X CHOH), 64.84, 63.95 (C-N), 53.75, 52.68, 50.68, 43.71, 39.71, 38.67, 38.42, 37.44, 36.13, 35.79, 31.59, 30.92, 28.59, 26.70, 23.23, 23.05, 21.09, 21.01, 20.16, 17.76, 14.09, 12.05, 9.41; exact mass: 607.3981 (607.3985 calcd for $C_{36}H_{53}N_3O_5$).

(22*E*)-3β-Acetoxycholesta-5,22-diene (70)[282]

The method[249] for conversion of **3** to **5** was used. Thus, **9** (810 mg, 2.10 mmol) gave 893 mg (quantitative) of pure **70** which was recrystallised from EtOH: mp 120 - 122 °C, (lit.[282] mp 125 - 128 °C); $[\alpha]_D^{24}$ -58.9° (*c* 2.07, $CHCl_3$), (lit.[282] $[\alpha]_D^{24}$ -61° ($CHCl_3$)); IR ($CHCl_3$ cast) 2947, 2865 (C-H), 1728 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.38 (d, 4.4 Hz, m, 1H, CH=C), 5.27 (AB, 15.2 Hz, d, 7.8 Hz, 1H, 22-HC=CH), 5.23 (AB, 15.2 Hz, dd, 6.4, 6.4 Hz, 1H, 23-HC=CH), 4.60 (m, 1H, CHOAc), 2.33 (m, 2H, CH, CH_2), 2.04 (s, 3H, CH_3CO_2), 0.9-2.1 (m, 22H, CH, CH_2), 1.020 (s, 3H, CH_3-19), 1.010 (d, ≈7 Hz, 3H, CH_3-21), 0.857 (d, 6.8 Hz, 3H, CH_3-26), 0.855 (d, 6.6 Hz, 3H, CH_3-27), 0.692 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.38 (C=O), 139.73 (wk, C=CH), 138.09, 126.27 and 122.60 (CH=C, CH=CH), 73.99 (CHOAc), 56.84, 56.03, 50.12, 42.29, 41.98, 40.05, 39.70, 38.18, 37.06, 36.65, 31.94, 31.92, 28.58, 28.58, 27.83, 24.30, 22.28, 22.25, 21.35, 21.06, 20.87, 19.30, 12.06; exact mass: 366.3286 (366.3286 calcd for $C_{27}H_{42}$, M^+ - AcOH). Anal. calcd for $C_{29}H_{46}O_2$: C, 81.63; H, 10.87. Found: C, 81.74; H, 10.64.

(22*E*)-3β-Acetoxycholesta-5,7,22-triene N-phenyltriazoline-3,5-dione adduct (71)

The method for conversion of **5** to **26** was used. Thus, **70** (362 mg, 848 μmol) afforded 223 mg of partially purified adduct **71**. Further purification was effected by preparative HPLC (Whatman Partisil 10 magnum column, EtOAc 25%/hexane 75%) or by MPLC (SiO_2, EtOAc 40%/hexane 60%) to give 150 mg (30%) of **71** as a gum: $[\alpha]_D^{24}$ - 104.5° (*c* 1.07, $CHCl_3$); IR ($CHCl_3$ cast) 2955, 2870 (C-H), 1755 (C=O amide), 1735 (C=O ester), 1704 (C=O amide), 1397, 1280, 750 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.45 (m, 5H, ArH), 6.42, 6.26 (AB, 8.0 Hz, 2H, CH=CH (ring)), 5.50 (dddd, 11.2, 11.2, 5.2, 5.2 Hz, 1H, CHOAc), 5.30 (m, 2H, CH=CH (chain)), 3.28 (dd,13.6, 5.0 Hz, 1H, 3α-H), 1.2-2.7 (m, 20H, CH, CH_2), 2.08 (s, 3H, CH_3CO_2), 1.025 (d, 6.5 Hz, 3H, CH_3-21), 0.985 (s, 3H, CH_3-19), 0.850 (d, 6.5 Hz, 3H, CH_3-26), 0.845 (d, 6.5 Hz, 3H, CH_3-27), 0.812 (s, 3H, CH_3-18); ^{13}C NMR (50 MHz, $CDCl_3$) δ 169.65 (C=O), 148.87 and 146.33 (wk, 2 X C=O amide), 137.15, 134.95, 128.92, 127.42 and 126.67 (CH=CH (ring), CH=CH (chain), *para* -ArC), 131.62 (wk, *ipso* -ArC), 128.52 and 125.96 (4C, *ortho* -ArC, *meta* -ArC), 70.28 (CHOAc), 65.10, 64.70, 54.84, 52.66, 49.13, 43.66, 41.72, 40.87, 39.29, 37.90, 33.47, 30.76, 28.28, 27.43, 25.74, 23.13, 22.42, 22.13, 22.03, 21.04, 17.24, 13.15, 13.03; MS (NH_3-CI) m/e 425 (MH^+ - **24**, 6%), 365 (MH^+ - **24** - AcOH, 100%).

6,7-Dihydro-(3β-acetoxycholesta-5,7-diene N-phenyltriazoline-3,5-dione adduct) (27) from 71

The method[256] used to convert **26** to **27** was used. Thus, **71** (10.6 mg, 17.9 μmol) afforded 10.6 mg (98%) of **27** which was identical (^1H NMR, ^{13}C NMR, exact mass) to **27** obtained from **26**.

(22*E*)-Cholesta-5,7,22-trien-3β-ol (7)[287]

The method[253] used to convert **26** to **6** was used. Thus, **71** (137 mg, 228 μmol) afforded 60.2 mg (69%) of **7** which was recrystallised from acetone: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.58 (dd, 5.2, 2.2 Hz, 1H, C=CH), 5.39 (ddd, 5.6, 2.8, 2.8 Hz, 1H, C=CH), 5.29 (AB, 15.2 Hz, dd, 6.6, 6.6 Hz, 1H, 23-HC=CH), 5.25 (AB, 15.0 Hz, d, 8.0 Hz, 1H 22-HC=CH), 3.64 (dddd, 11.4, 11.4, 4.4, 4.4 Hz, 1H, CHOH), 2.48 (AB, 14.0 Hz, dd, 4.8, 2.0 Hz, 1H, CHH), 2.28 (AB, ≈13 Hz, d, 12.8 Hz, br, 1H, CHH), 1.2-2.2 (m, 20H, CH, CH_2), 1.035 (d, 6.8 Hz, 3H, CH_3-21), 0.945 (s, 3H, CH_3-19), 0.867 (d, 6.8 Hz, 3H, CH_3-26), 0.862 (d, 6.8 Hz, 3H, CH_3-27), 0.632 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.26 and 139.86 (wk, C=CH-CH=C), 137.83 and 126.54 (CH=CH (chain)), 119.64 and 116.39 (C=CH-CH=C), 70.51 (CHOH), 55.86, 54.65, 46.41, 42.94, 41.98, 40.92, 40.26, 39.22, 38.46, 37.13, 32.11, 29.68, 28.57, 28.32, 23.04, 22.25, 21.21, 20.98, 16.32, 12.03.

Trifluorodiazoethane (76)[353]

The method of Gilman and Jones[353] was used. To a mixture of 2,2,2-trifluoroethylamine hydrochloride (6.50 g, 48.0 mmol), H_2O (25 mL) and CH_2Cl_2 (37.5 mL) in a strong round-bottom flask was added sodium nitrite (3.75 g, 54.3 mmol). The flask was stoppered, shaken for 5 min, and cooled to -15 °C. The organic layer was collected, and the aqueous layer was extracted (CH_2Cl_2, 2 X 37.5 mL). The combined organic extracts were washed (10% Na_2CO_3, 100 mL) and dried over $CaCl_2$ at -10 °C for 16 h. The solution was distilled to afford 74 mL of a yellow CH_2Cl_2 solution of **76**: IR (CH_2Cl_2 cast) 2118 ($C=N^+=N^-$), 1393, 1238, 1165, 1148, 1106 cm^{-1}; 6.85 mL of solution was required to decolorize 0.670 g of iodine in Et_2O (20 mL): 0.385 M in **76**, equivalent to 59% yield.

2-Diazo-3,3,3-trifluoropropionyl chloride (75)[341]

The method of Chowdhry *et al*[341] was followed. To a solution of **76** in CH_2Cl_2 (0.385 M, 45 mL, 17.34 mmol) was added dipotassium hydrogen phosphate (8 g) and a solution of phosgene (4.5 mL) in CH_2Cl_2 (5 mL). The mixture was stirred for 16 h at room temperature, filtered, and the solvent was removed by distillation through a Vigreux column. The residue was distilled to afford 370 mg (13%) of **75** as a yellow liquid: bp 45 - 50 °C, at 200 mm Hg, (lit.[341] bp 59 - 60 °C at 137 mm Hg); IR ($CHCl_3$ cast) 2144 ($C{=}N^+{=}N^-$), 1750 (C=O), 1330, 1159 cm^{-1}; ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.62 (br, v wk, $\underline{C}{=}O$), 121.23 (q, $^1J_{(^{13}C\text{-}^{19}F)}$= 275 Hz, $\underline{C}F_3$), 71.78 (br, v wk, $C{=}N^+{=}N^-$); ^{19}F NMR (376 MHz, $CDCl_3$) δ -65.05 (br, $C\underline{F}_3$); exact mass: 171.9644 (171.9651 calcd for $C_3N_2OF_3{}^{35}Cl$).

3β-(2'-Diazo-3', 3', 3'-Trifluoropropionyloxy)-cholest-5-ene (72)

To a solution of **3** (261 mg, 674 μmol) in THF (5 mL) was added a solution of n-butyllithium (1.60 M in hexane, 420 μL, 627 μmol). The mixture was stirred for 10 min, and a solution of *p* -nitrophenyl 3,3,3-trifluoro-2-diazopropionate (200 mg, 727 μmol) in THF (5 mL) was added rapidly. The mixture was stirred for 30 min, then H_2O (0.5 mL) was added and the mixture was concentrated *in vacuo* . Aqueous 1M HCl (20 mL) was added, and the mixture was extracted ($CHCl_3$, 2 X 20 mL). The extracts were dried and concentrated *in vacuo* . Column chromatography (EtOAc 4%/hexane 96%) gave 281 mg (80%) of **72** as a very pale yellow solid: mp 90 °C dec.; $[\alpha]_D{}^{24}$ -20.5° (*c* 2.21, $CHCl_3$); IR ($CHCl_3$ cast) 2960, 2925, 2895, 2860 (C-H), 2135 ($C{=}N^+{=}N^-$), 1715 (C=O), 1324, 1052 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.39 (br d, 5.2 Hz, $C\underline{H}{=}C$), 4.76 (dddd, 10.7, 10.7, 6.2, 4.5 Hz, 1H, $C\underline{H}OR$), 2.38 (m, 2H, 4-$C\underline{H}_2$), 1.75-2.10 (m, 5H, $C\underline{H}$, $C\underline{H}_2$), 0.8-1.7 (m, 21H, $C\underline{H}$, $C\underline{H}_2$), 1.025 (s, 3H, $C\underline{H}_3$-19), 0.915 (d, 6.2 Hz, 3H, $C\underline{H}_3$-21), 0.866 (d, 7.0 Hz, 3H, $C\underline{H}_3$-26), 0.864 (d, 6.7 Hz, 3H, $C\underline{H}_3$-27), 0.680 (s, 3H, $C\underline{H}_3$-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.61 (v wk, $\underline{C}{=}O$), 139.57 (wk,

C=CH), 123.43 (CH=C), 123.08 (wk, q, $^{1}J_{(^{13}C-^{19}F)}$= 270 Hz, CF_3), 76.33 (CHOR), 56.75, 56.31, 50.11, 42.30, 39.71, 39.45, 38.03, 36.83, 36.48, 36.13, 35.64, 31.79, 31.79, 31.37 (v wk, C=N_2), 27.99, 27.81, 27.70, 24.09, 23.71, 22.48, 22.37, 22.27, 20.89, 19.01, 18.51, 11.58; ^{19}F NMR (376 MHz, $CDCl_3$) δ -57.62 (br s, $w_{1/2}$ = 40 Hz, CF_3); UV (dioxan) λ_{max} (logε) 232 (4.066), 236 (4.095); MS (EI) m/e 522 (M^+, 0.1%), 494 ($M^+ - N_2$, 0.2%), 368 ($M^+ - CF_3CN_2CO_2H$, 100%); MS (NH_3-CI) m/e 540 ($[M + NH_4]^+$, 35%), 512 ($[M + NH_4]^+ - N_2$, 14%). Anal. calcd for $C_{30}H_{45}O_2N_2F_3$: C, 68.94; H, 8.68; N, 5.36. Found: C, 69.37; H, 8.66; N, 5.61.

(20*S*)-20-(2'-Diazo-3',3',3'-trifluoropropionyloxymethyl)-6β-methoxy-3α,5-cyclo-5α-pregnane (74)

To a solution of **46** (99.6 mg, 288 μmol) in THF (4 mL) at -78 °C was added a solution of n-butyllithium (1.60 M in hexane, 185 μL, 296 μmol). The mixture was stirred for 15 min, warmed slowly to room temperature and stirred for 40 min, and concentrated *in vacuo*. Column chromatography ($CHCl_3$) then a second column (EtOAc 4%/hexane 96%) afforded 41.6 mg (24%) of **74** as a clear oil: $[\alpha]_D^{24}$ +38.2° (*c* 2.09, $CHCl_3$); IR ($CHCl_3$ cast) 2980 (C-H), 2130 (C=N^+=N^-), 1730 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.26 (AB, 10.4 Hz, d, 3.2 Hz, 1H, CHHOCO), 3.98 (AB, 10.4 Hz, d, 7.2 Hz, 1H, CHHOCO), 3.31 (s, 3H, CH_3O), 2.76 (dd, 2.7, 2.7 Hz, 1H, CHOMe), 0.76-2.00 (m, 20H, CH, CH_2), 1.024 (s, 3H, CH_3-19), 1.019 (d, 6.6 Hz, 3H, CH_3-21), 0.744 (s, 3H, CH_3-18), 0.64 (dd, 4.8, 3.8 Hz, 1H, 4-H), 0.42 (dd, 7.8, 5.0 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.01 (C=O), 122.77 (v wk, q, $^{1}J_{(^{13}C-^{19}F)}$= 269 Hz, CF_3), 82.43 (CHOMe), 71.18 (CH_2O), 56.51, 56.28, 52.85, 48.09, 43.44, 43.01, 40.18, 35.96, 35.40, 35.11, 33.44, 30.58, 27.68, 24.97, 24.25, 22.76, 21.55, 19.22, 16.93, 13.09, 12.27, C=N_2 not observed; ^{19}F NMR (376 MHz, $CDCl_3$) δ -57.47 (br s, $w_{1/2}$ =50 Hz, CF_3); MS (NH_3-CI) m/e 482 (M^+, 2%).

A similar reaction of **46** with 2-diazo-3,3,3-trifluoropropionyl chloride (**75**) in

place of *p* -nitrophenyl 2-diazo-3,3,3-trifluoropropionate afforded 20% of **74**.

(20*S*)-20-(2'-Diazo-3',3',3'-trifluoropropionyloxymethyl)-pregn-5-en-3β-ol (73)

The method[267] used for conversion of **35** to **8** was used. Thus, **74** (20.9 mg, 43.3 μmol) afforded 18.8 mg (93%) of **73** as a gum: $[\alpha]_D^{24}$ -34.1° (*c* 1.88, $CHCl_3$); IR ($CHCl_3$ cast) 3360 (br, O-H), 2962, 2937, 2903, 2870 (C-H), 2135 (C=N^+=N^-), 1728 (C=O), 1350, 1321, 1137, 1060 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.37 (br d, 5.2 Hz, 1H, C<u>H</u>=C), 4.27 (A<u>B</u>, 10.8 Hz, d, 3.4 Hz, 1H, C<u>H</u>HOCO), 3.98 (A<u>B</u>, 10.6 Hz, d, 7.2 Hz, 1H, CH<u>H</u>OCO), 3.52 (dddd, 11.2, 11.2, 4.0, 4.0 Hz, 1H, C<u>H</u>OH), 2.29 (m, 2H, 4-$C\underline{H}_2$), 2.00 (m, 2H, C<u>H</u>, $C\underline{H}_2$), 1.84 (m, 4H, C<u>H</u>, $C\underline{H}_2$), 0.9-1.7 (m, 14H, C<u>H</u>, $C\underline{H}_2$), 1.03 (d, 6.4 Hz, 3H, $C\underline{H}_3$-21), 1.01 (s, 3H, $C\underline{H}_3$-19), 0.71 (s, 3H, $C\underline{H}_3$-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.50 (v wk, <u>C</u>=O), 141.11 (wk, <u>C</u>=CH), 122.95 (v wk, q, $^1J_{(^{13}C\text{-}^{19}F)}$= 270 Hz, <u>C</u>$F_3$), 121.75 (C=<u>C</u>H), 71.73 and 71.12 (<u>C</u>HOH, $\underline{C}H_2OCO$), 56.35, 52.47, 49.97, 42.38, 42.15, 39.43, 37.09, 36.31, 35.76, 31.73, 31.62, 31.45, 27.38, 24.09, 20.80, 19.10, 16.66, 11.57. <u>C</u>=N^+=N^- not observed; ^{19}F NMR (376 MHz, $CDCl_3$) δ -57.80 (br s, $w_{1/2}$ ≈50 Hz, $C\underline{F}_3$).

α,α-Diethoxytoluene (78)

The method of Evans[359] was used. A mixture of benzaldehyde (43.0 g, 406 mmol), triethyl orthoformate (67.1 g, 230 mmol) and Amberlyst™ 15 ion exchange resin (2 g) in EtOH (200 mL) was heated at reflux for 3 h, then stirred at room temperature for 16 h. The mixture was filtered. Solvent was removed from the filtrate, which was distilled to afford 72.5 g (89%) of **78** as a clear liquid: bp 97-99 °C at 18-22 mm Hg; IR ($CHCl_3$ cast) 3085-3030 (wk, Ar-H), 2976, 2925, 2880 (C-H), 1205 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.40-7.52 (m, 2H, Ar<u>H</u>), 7.20-7.38 (m, 3H, Ar<u>H</u>), 5.50 (s, 1H, C<u>H</u>$(OEt)_2$), 3.56 (AB, 9.3 Hz, q, 6.8 Hz, 4H, 2 X $C\underline{H}_2O$), 1.12 (t, 6.8 Hz, 6H, 2 X $C\underline{H}_3$): the AB

assignment at δ 3.56 was confirmed by recording the spectrum at 360 MHz, and by decoupling of the CH_3 groups; ^{13}C NMR (50 MHz, $CDCl_3$) δ 139.03 (wk, *ipso* -ArC), 128.03 (*para* -ArC), 127.94 and 126.49 (4C, *ortho* -ArC, *meta* -ArC), 101.35 ($CH(OEt)_2$), 60.72 (2 X CH_2O), 15.02 (2 X CH_3); exact mass: 180.1147 (180.1150 calcd for $C_{11}H_{16}O_2$).

N-Benzylidinebenzenesulfonamide (79)[356, 396]

The method of Davis *et al*[356] was followed. A mixture of benzenesulfonamide (52.3 g, 330 mmol) and **78** (59 g, 330 mmol) was heated on an oil bath at 150-170 °C; the evolved EtOH was collected until 37 mL had been received (30 min). Residual EtOH was removed from the hot material *in vacuo*. The white solid obtained upon cooling was dissolved in boiling CH_2Cl_2 (100 mL), and pentane (400 mL) added. Compound **79** (73.5 g (91%)) was collected as a white solid: mp 76 - 77 °C, (lit.[396] mp 80 °C); IR ($CHCl_3$, cast) 1597, 1571, 1445 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 9.08 (s, 1H, CH=N), 7.86-8.08 (m, 4H, ArH), 7.40-7.70 (m, 6H, ArH); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.55 (C=N), 134.98 and 133.47 (2 X *para* -ArC), 131.27, 127.94, 129.10 and 129.10 (8C, 2 X *ortho* -ArC, 2 X *meta* -ArC), 132.00 and 132.52 (v wk, 2 X *ipso* -ArC); exact mass: 245.0512 (245.0511 calcd for $C_{13}H_{11}NO_2S$).

2-Benzenesulfonyl 3-phenyloxaziridine (77)[397]

The method of Davis *et al*[397] was used. Thus, $NaHCO_3$ (22.0 g, 272 mmol) and tetra-n-butylammonium bisulfate (2.06 g) were added to a solution of **79** (24.5 g, 100 mmol) in $CHCl_3$ (320 mL) and H_2O (240 mL). The mixture was cooled to 0 °C, and stirred vigourously (mechanical stirrer). A solution of *meta* -chloroperoxybenzoic acid (21.0 g, 103 mmol) in $CHCl_3$ (250 mL) was added dropwise over 30 min, and the mixture was stirred at 0 °C for 15 min. The organic phase was separated, washed (H_2O, 200 mL, 10% w/v Na_2SO_3, 200 mL, and H_2O, 200 mL), dried over K_2CO_3 and concentrated *in*

vacuo. Filtration through silica and elution with CH_2Cl_2 (1.5 L) afforded 20.8 g (80%) of **77** as a white unstable solid: IR ($CHCl_3$ cast) 3060 (wk, Ar-H), 1450, 1353, 1184, 1172 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.3-8.1 (m, 10H, ArH), 5.46 (s, 1H, CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 134.98 and 131.39 (2 X *para* -ArC), 134.60, 130.38 (wk, 2 X *ipso* -ArC), 129.34, 129.29, 128.70 and 128.11 (8C, 2 X *ortho* -ArC, 2 X *meta* -ArC), 76.26 (CH(O)N); exact mass: 261.0463 (261.0459 calcd for $C_{13}H_{11}NO_3S$).

6β-Hydroxycholest-4-en-3-one (80)[398]

A modification of the enolate oxidation procedure of Davis *et al*[356, 397] was used. To a solution of diisopropylamine (34 μL, 260 μmol) in THF (5 mL) at 0 °C was added n-butyllithium (1.60 M in hexane, 170 μL, 260 μmol). The mixture was stirred for 15 min, and cooled to -78 °C. A solution of cholest-4-en-3-one (99.9 mg, 259 μmol) in THF (3 mL) was added. After 20 min, a solution of **77** (81.7 mg, 313 μmol) in THF (2 mL) was added dropwise over 1 min. The mixture was warmed to room temperature over 1 h and H_2O (1 mL) added. The mixture was acidified with 1 M HCl, and THF was removed on the rotary evaporator. H_2O (10 mL) was added, and the mixture was extracted ($CHCl_3$, 2 X 10 mL). The extracts were dried and concentrated *in vacuo*. Column chromatography gave 20 mg of material that eluted in MeOH 2%/$CHCl_3$ 98%, after elution of starting material and aromatic products with $CHCl_3$. Repeated column chromatography (MeOH 2%/$CHCl_3$ 98%) afforded 8.6 mg (8%) of ca. 90% pure **80** as a gum: IR ($CHCl_3$ cast) 3440 (br, O-H), 2945, 2935, 2862 (C-H), 1680 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.84 (s, CH=C), 4.36 (dd, 3.0, 3.0 Hz, 1H, CHOH), 2.55 (AB, 17.2 Hz, dd, 14.8, 5.2 Hz, 1H, 2-H), 2.40 (AB, 17.6 Hz, m, 1H, 2-H), 0.8-2.15 (m, CH, CH_2), 1.38 (s, 3H, CH_3-19), 0.92 (d, CH_3-21), 0.88 (2 X d, CH_3-26, CH_3-27), 0.74 (s, 3H, CH_3-18); exact mass: 400.3333 (400.3342 calcd for $C_{27}H_{44}O_2$).

3β-Acetoxy-7-oxocholest-5-ene (81)[240, 360]

The method of Dauben and Fonken[360] was followed. To a solution of **5** (10.8 g, 23.2 mmol) in AcOH (120 mL) at 55 °C was added a solution of CrO_3 (7.0 g, 70 mmol) in AcOH 50%/H_2O 50% (20 mL) dropwise over 2 h, while maintaining the temperature at 50-60 °C. The mixture was stirred at this temperature for 2 h, and MeOH (6 mL) was added. AcOH (80 mL) was removed by vacuum distillation. The mixture was cooled, and H_2O (5 mL) was added. After 3 h, the precipitate was collected and washed (AcOH 80%/H_2O 20%). Column chromatography (EtOAc 4%/hexane 96%, then EtOAc 20%/hexane 80%) afforded:

Fraction (1), recovered **5**.

Fraction (2), 1.77 g of white crystalline **81**; a second crop from the crude mother liquors afforded a further 0.36 g after similar work-up, for a total yield of 2.13 g (21%): mp 156 - 157 °C, (lit.[240] mp 153 - 154 °C); $[\alpha]_D^{24}$ -97.9° (*c* 2.06, $CHCl_3$); IR ($CHCl_3$ cast) 2949, 2871 (C-H), 1733 (C=O ester), 1671 (C=O), 1467, 1246 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.68 (d, 1.2 Hz, 1H, C<u>H</u>=C), 4.71 (m, 1H, C<u>H</u>OAc), 2.1-2.6 (m, 4H, C<u>H</u>, C<u>H</u>$_2$), 2.04 (s, 3H, C<u>H</u>$_3$CO$_2$), 0.9-2.1 (m, 22H, C<u>H</u>, C<u>H</u>$_2$), 1.210 (s, 3H, C<u>H</u>$_3$-19), 0.895 (d, 6.4 Hz, 3H, C<u>H</u>$_3$-21), 0.860 (d, 6.0 Hz, 3H, C<u>H</u>$_3$-26), 0.855 (d, 6.0 Hz, 3H, C<u>H</u>$_3$-27), 0.655 (s, 3H, C<u>H</u>$_3$-18); ^{13}C NMR (75 MHz, $CDCl_3$) δ 201.89 (wk, <u>C</u>=O), 170.24 (wk, <u>C</u>=O ester), 163.79 (wk, <u>C</u>=CH), 126.69 (C=<u>C</u>H), 72.19 (<u>C</u>HOAc), 54.76, 49.94, 49.80, 45.40, 43.09, 39.45, 38.65, 38.29, 37.72, 36.16, 35.98, 35.69, 28.51, 27.97, 27.34, 26.28, 23.80, 22.78, 22.53, 21.23, 21.15, 18.84, 17.23, 11.94; MS (EI) m/e 442 (M^+, 2%), 382 (M^+ - AcOH, 100%). Anal. calcd for $C_{29}H_{46}O_3$: C, 78.68; H, 10.48. Found: C, 78.43; H, 10.44.

7-Oxocholest-5-en-3β-ol (82)[361]

Potassium *t*-butoxide (37.0 mg, 303 μmol) was added to a solution of **81** (111 mg, 250 μmol) in THF (5 mL) and H_2O (3 mL). The mixture was stirred for 7 h, then

acidified with HCl and diluted with H_2O (50 mL). The mixture was extracted (CH_2Cl_2, 2 X 50 mL), and the extracts were dried and concentrated *in vacuo* to afford 99.2 mg (99%) of crystalline **82**: mp 165 - 166 °C, (lit.[361] mp 168 - 170 °C); $[\alpha]_D^{24}$ -105° (*c* 1.19, $CHCl_3$), (lit.[361] $[\alpha]_D^{19}$ -106° (*c* 0.8, $CHCl_3$)); IR ($CHCl_3$ cast) 3520 (O-H), 2940, 2865 (C-H), 1664 (C=O), 1465, 1382 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.68 (d, 1.2 Hz, 1H, C<u>H</u>=C), 3.67 (m, 1H, C<u>H</u>OH), 2.30-2.55 (m, 4H, C<u>H</u>, C<u>H</u>$_2$), 2.25 (dd, 14, 14 Hz, 1H, C<u>H</u>CO), 1.8-2.1 (m, 4H, C<u>H</u>, C<u>H</u>$_2$), 0.95-1.75 (m, 18H, C<u>H</u>, C<u>H</u>$_2$), 1.200 (s, 3H, C<u>H</u>$_3$-19), 0.920 (d, 6.5 Hz, 3H, C<u>H</u>$_3$-21), 0.870 (d, 6.5 Hz, 3H, C<u>H</u>$_3$-26), 0.863 (d, 6.5 Hz, 3H, C<u>H</u>$_3$-27), 0.683 (s, 3H, C<u>H</u>$_3$-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.42 (wk, <u>C</u>=O), 165.52 (wk, <u>C</u>=CH), 125.92 (<u>C</u>H=C), 70.33 (<u>C</u>HOH), 54.75, 49.92, 49.89, 45.35, 43.05, 41.79, 39.41, 38.65, 38.25, 36.32, 36.13, 35.65, 31.07, 28.48, 27.92, 26.26, 23.78, 22.75, 22.50, 21.16, 18.82, 17.25, 11.91; exact mass: 400.3341 (400.3341 calcd for $C_{27}H_{44}O_2$). Anal. calcd for $C_{27}H_{44}O_2$: C, 80.94; H, 11.07. Found: C, 81.23; H, 11.19.

3β-(*t*-Butyldimethylsilyloxy)-7-oxo-cholest-5-ene (83)

The method of Hosoda *et al* [362] was used. A mixture of *t*-butyldimethylsilyl chloride (125 mg, 829 μmol) and imidazole (142 mg, 2.09 mmol) were added to a solution of **82** (88.9 mg, 222 μmol) in DMF (2 mL). The mixture was shaken for 5 min, and allowed to stand for 20 min. Et_2O (25 mL) was added, and the mixture was washed (H_2O, 3 X 25 mL). The organic phases were dried, and concentrated *in vacuo*. Column chromatography (EtOAc 4%/hexane 96%) to remove *t*-butyldimethylsilyl chloride afforded 98.6 mg (86%) of **83** as a white solid: mp 214 - 215 °C; $[\alpha]_D^{24}$ -78.4° (*c* 2.07, $CHCl_3$); IR ($CHCl_3$ cast) 2948, 2936, 2860 (C-H), 1667 (C=O), 1254, 836, 774 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.68 (br s, $w_{1/2}$ 3 Hz, 1H, C<u>H</u>=C), 3.62 (m, 1H, C<u>H</u>OTBDMS), 2.15-2.55 (m, 4H, C<u>H</u>, C<u>H</u>$_2$), 1.75-2.10 (m, 4H, C<u>H</u>, C<u>H</u>$_2$), 1.0-1.7 (m, 18H, C<u>H</u>, C<u>H</u>$_2$), 1.190 (s, 3H, C<u>H</u>$_3$-19), 0.920 (d, 6.3 Hz, 3H, C<u>H</u>$_3$-21), 0.885 (s, 9H,

$(CH_3)_3CSi$), 0.860 (d, 6.3 Hz, 3H, CH_3-26), 0.855 (d, 6.3 Hz, 3H, CH_3-27), 0.670 (s, 3H, CH_3-18), 0.055 (s, 6H, $(CH_3)_2Si$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 202.24 (wk, C=O), 165.76 (wk, C=CH), 125.85 (CH=C), 71.37 (CHOTBDMS), 54.91, 50.12, 50.07, 45.46, 43.14, 42.59, 39.51, 38.81, 38.37, 36.49, 36.24, 35.72, 31.80, 28.55, 28.01, 26.34, 25.84 (3C, $(CH_3)_3CSi$), 23.86, 22.79, 22.54, 21.25, 18.90, 18.13 (wk, Me_3CSi), 17.32, 11.99, -4.63 (2C, $(CH_3)_2Si$); MS (EI) m/e 514 (M^+, 0.8%); MS (NH_3-CI) m/e 515 (MH^+, 100%); UV (cyclohexane) λ_{max} (logε) 231 (4.129), 267 (2.655). Anal. calcd for $C_{33}H_{58}O_2Si$: C, 76.98; H, 11.35. Found: C, 77.14; H, 11.45.

7β-Hydroxy-3β-(*t* -butyldimethylsilyloxy)-cholest-5-ene (84) and 7α-hydroxy-3β-(*t* -butyldimethylsilyloxy)-cholest-5-ene (85)

The reduction procedure of Mosbach *et al* [363, 364] was modified. Thus, $NaBH_4$ (250 mg) was added to a solution of **83** (1.46 g, 2.84 mmol) in THF (50 mL) and methanol (35 mL). The mixture boiled, and was stirred for 20 min. H_2O (200 mL) was added and the mixture was acidified with HCl. After most of the MeOH and THF had been removed on the rotary evaporator, the mixture was extracted ($CHCl_3$, 3 X 200 mL). The extracts were washed (H_2O, 200 mL), dried and concentrated *in vacuo*. ^1H NMR showed a ratio of **85**:**84** of 17:83. Column chromatography (EtOAc 4%/hexane 96%) gave:

Fraction (1), 168 mg (11%) of **85** as a white solid: mp 165 - 167 °C; $[\alpha]_D^{24}$ - 58.0° (*c* 1.15, $CHCl_3$); IR ($CHCl_3$ cast) 3430 (O-H), 2949, 2932, 2902, 2857 (C-H), 1255, 1100, 832, 772 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.57 (dd, 5.3, 1.6 Hz, 1H, CH=C), 3.85 (br s, $w_{1/2}$= 11.5 Hz, 1H, CHOH), 3.53 (dddd, 10.8, 10.8, 5.0, 5.0 Hz, 1H, CHOTBDMS), 2.31 (AB, 13.3 Hz, ddd, 11.0, 1.7, 1.7 Hz, 1H, 4β-H), 2.20 (AB, 13.5 Hz, dd, 5.2, 2.0 Hz, 1H, 4α-H), 0.95-2.10 (m, 25H, CH, CH_2), 0.985 (s, 3H, CH_3-19), 0.925 (d, 6.5 Hz, 3H, CH_3-21), 0.887 (s, 9H, $(CH_3)_3CSi$), 0.866 (d, 6.5 Hz, 3H, CH_3-26), 0.862 (d, 6.5 Hz, 3H, CH_3-27), 0.680 (s, 3H, CH_3-18), 0.057 (s, 6H, $(CH_3)_2Si$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 146.95 (C=CH), 123.30 (C=CH), 72.09

(CHOTBDMS), 65.38 (CHOH), 55.79, 49.39, 42.54, 42.23, 42.09, 39.48, 39.17, 37.48, 37.42, 37.05, 36.13, 35.72, 31.79, 28.24, 27.97, 25.86 (3C, $(CH_3)_3CSi$), 24.26, 23.65, 22.77, 22.53, 20.64, 18.70 (wk, Me_3CSi), 18.61, 18.21, 11.58, -4.63 (2C, $(CH_3)_2Si$); MS (NH_3-CI) m/e 516 (M^+, 0.6%), 499 ($MH^+ - H_2O$, 68%), 367 ($MH^+ - H_2O$ - TBDMSOH, 100%). Anal. calcd for $C_{33}H_{60}O_2Si$: C, 76.68; H, 11.70. Found: C, 77.02; H, 11.72.

Fraction (2), 347 mg (24%) of fractions containing a mixture of **84** and **85**.

Fraction (3), 979 mg (67%) of **84** as a white solid: mp 130 - 131 °C; $[\alpha]_D^{24}$ +0.2° (*c* 1.75, $CHCl_3$); IR ($CHCl_3$ cast) 3342 (O-H), 2952, 2933, 2902, 2858 (C-H), 1472, 1462, 1205, 1099, 837, 776 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.25 (dd, 1.8, 1.8 Hz, 1H, CH=C), 3.83 (br d, 7.2 Hz, 1H, CHOH), 3.50 (dddd, 10.7, 10.7, 4.8, 4.8 Hz, 1H, CHOTBDMS), 2.28 (AB, 13.3 Hz, ddd, 11.0, 2.0, 2.0 Hz, 1H, 4β-H), 2.21 (AB, 13.5 Hz, dd, 5.2, 1.8 Hz, 4α-H), 2.02 (ddd, 12.7, 3.3, 3.3 Hz, 1H, CH, CH_2), 0.90-1.95 (m, 24H, CH, CH_2), 1.038 (s, 3H, CH_3-19), 0.920 (d, 6.5 Hz, 3H, CH_3-21), 0.886 (s, 9H, $(CH_3)_3CSi$), 0.868 (d, 6.6 Hz, 3H, CH_3-26), 0.863 (d, 6.6 Hz, 3H, CH_3-27), 0.686 (s, 3H, CH_3-18), 0.057 (s, 6H, $(CH_3)_2Si$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 144.21 (wk, C=CH), 125.05 (CH=C), 73.44 and 72.27 (CHOTBDMS, CHOH), 56.02, 55.48, 48.35, 42.93, 42.29, 40.96, 39.61, 39.51, 37.07, 36.51, 36.22, 35.72, 32.05, 29.59, 28.55, 28.01, 26.39, 25.91 (3C, $(CH_3)_3CSi$), 23.83, 22.81, 22.55, 21.06, 19.17, 18.78, 18.22 (wk, Me_3CSi), 11.82, -4.59 (2C, $(CH_3)_2Si$); MS (NH_3-CI) m/e 516 (M^+, 0.7%), 499 ($MH^+ - H_2O$, 55%), 367 ($MH^+ - H_2O$ - TBDMSOH, 100%). Anal. calcd for $C_{33}H_{60}O_2Si$: C, 76.68; H, 11.70. Found: C, 76.61; H, 11.64.

Alcohols 84 and 85 by reduction of 83 with lithium aluminium hydride

Ketone **83** (40.3 mg, 91.0 μmol) in THF (5 mL) was treated with lithium aluminium hydride (100 mg), and the mixture was stirred for 30 min. EtOAc, then H_2O,

then 1 M HCl (until acidic) were added to destroy excess hydride. The mixture (50 mL) was extracted with $CHCl_3$ (3 X 20 mL), and the extracts were dried and concentrated *in vacuo* to give a mixture of **84** and **85** in a ratio of 26:74, as determined by ^1H NMR.

7α-Acetoxy-3β-(*t*-butyldimethylsilyloxy)-cholest-5-ene (87)

The method of Staunton and Eisenbraun[249] was used. A mixture of **84** (156 mg, 302 μmol), acetic anhydride (1.5 mL) and pyridine (3 mL) was stirred for 20 h. H_2O (25 mL) was added, and the mixture was acidified with 1 M HCl and extracted ($CHCl_3$, 3 X 25 mL). The extracts were washed (saturated $CuSO_4$), dried and concentrated *in vacuo*. Column chromatography (EtOAc 4%/hexane 96%) gave 159 mg (94%) of **87** as a gum: $[\alpha]_D^{24}$ -131.2° (*c* 2.91, $CHCl_3$); IR ($CHCl_3$ cast) 2952, 2935, 2900, 2865, 2855 (C-H), 1752 (C=O), 1242, 1095, 835 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.51 (dd, 5.0, 1.2 Hz, 1H, C<u>H</u>=C), 4.94 (ddd, 5.0, 5.0, 1.2 Hz, 1H, C<u>H</u>OAc), 3.50 (m, 1H, C<u>H</u>OTBDMS), 2.15-2.35 (m, 2H, 4-C<u>H</u>$_2$), 2.02 (s, 3H, C<u>H</u>$_3$CO$_2$), 1.0-2.1 (m, 24H, C<u>H</u>, C<u>H</u>$_2$), 0.990 (s, 3H, C<u>H</u>$_3$-19), 0.920 (d, 6.4 Hz, 3H, C<u>H</u>$_3$-21), 0.880 (s, 9H, (C<u>H</u>$_3$)$_3$CSi), 0.860 (2 X d, obscure, 6H, C<u>H</u>$_3$-26, C<u>H</u>$_3$-27), 0.660 (s, 3H, C<u>H</u>$_3$-18), 0.050 (s, 6H, (C<u>H</u>$_3$)$_2$Si); MS (NH_3-CI) m/e 499 (MH^+ - AcOH, 45%), 367 (MH^+ - AcOH - TBDMSOH, 100%). Anal. calcd for $C_{35}H_{62}O_3Si$: C, 75.20; H, 11.18. Found: C, 75.17; H, 11.01.

7β-Acetoxy-3β-(*t*-butyldimethylsilyloxy)-cholest-5-ene (86)

The method[249] for conversion of **85** to **87** was used. Thus, **84** (326 mg, 630 μmol) afforded 350 mg (99%) of **86** as a gum: $[\alpha]_D^{24}$ +51.1° (*c* 2.03, $CHCl_3$); IR ($CHCl_3$ cast) 2951, 2935, 2855 (C-H), 1735 (C=O), 1240, 1095, 835, 772 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 5.16 (br s, 1H, C<u>H</u>=C), 4.98 (ddd, 8.8, 1.6, 1.6 Hz, 1H, C<u>H</u>OAc), 3.46 (ddd, 10.4, 10.4, 5.2, 5.2 Hz, 1H, C<u>H</u>OTBDMS), 2.1-2.35 (m, 2H, 4-C<u>H</u>$_2$), 2.00 (s, 3H, C<u>H</u>$_3$CO$_2$), 0.9-1.9 (m, 24H, C<u>H</u>, C<u>H</u>$_2$), 1.005 (s, 3H, C<u>H</u>$_3$-19),

0.930 (d, 7 Hz, 3H, CH_3-21), 0.900 (s, 9H, $(CH_3)_3CSi$), 0.875 (2 X d, obscure, 6H, CH_3-26, CH_3-27), 0.705 (s, 3H, CH_3-18), 0.055 (s, 6H, $(CH_3)_2Si$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.56 (wk, C=O), 146.34 (wk, C=CH), 121.31 (CH=C), 76.00 (CHOH), 72.13 (CHOTBDMS), 55.67, 55.57, 48.38, 42.83, 42.23, 39.41, 39.41, 36.92, 36.55, 36.42, 36.08, 35.50, 31.89, 28.14, 27.78, 25.68 (3C, $(CH_3)_3CSi$), 25.01, 23.64, 22.48, 22.27, 21.31, 20.93, 18.80, 18.57, 17.90 (wk, Me_3CSi), 11.52, -5.10 (2C, $(CH_3)_2Si$); MS (NH_3-CI) m/e 499 (MH^+ - AcOH, 59%), 367 (MH^+ - AcOH - TBDMSOH, 100%). Anal. calcd for $C_{35}H_{62}O_3Si$: C, 75.20; H, 11.18. Found: C, 75.05; H, 11.06.

7α-Acetoxycholest-5-en-3β-ol (89)[369]

The method of Hosoda *et al*[362] was used. A solution of tetra-n-butylammonium fluoride in THF (1 M, 0.5 mL, 500 μmol) was added to **87** (142 mg, 254 μmol) in THF (4 mL). The mixture was stirred for 150 min, and concentrated *in vacuo*. H_2O (10 mL) was added and the mixture was extracted (EtOAc, 2 X 10 mL). The extracts were washed (H_2O, 20 mL), dried and concentrated *in vacuo*. Column chromatography ($CHCl_3$, then MeOH 2%/$CHCl_3$ 98%) gave 107 mg (95%) of **89** as a white solid: mp 58 - 60 °C (becomes gummy); $[\alpha]_D^{24}$ -163.6° (*c* 1.07, $CHCl_3$); IR ($CHCl_3$ cast) 3400 (O-H), 2940, 2869 (C-H), 1730 (C=O), 1375, 1243, 757 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.57 (d, 5.0 Hz, 1H, CH=C), 4.95 (dd, 4.4, 4.4 Hz, 1H, CHOAc), 3.57 (dddd, 10.8, 10.8, 5.0, 5.0 Hz, 1H, CHOH), 2.2-2.4 (m, 2H, 4-CH_2), 2.17 (br s, 1H, OH), 2.02 (m, 1H, CH, CH_2), 2.02 (s, 3H, CH_3CO_2), 1.75-1.90 (m, 3H, CH, CH_2), 1.00-1.65 (m, 20H, CH, CH_2), 1.000 (s, 3H, CH_3-19), 0.925 (d, 6.5 Hz, 3H, CH_3-21), 0.867 (d, 6.8 Hz, 3H, CH_3-26), 0.862 (d, 6.6 Hz, 3H, CH_3-27), 0.669 (s, 3H, CH_3-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.65 (wk, C=O), 147.79 (wk, C=CH), 11[illegible] (C=CH), 71.15

20.76, 18.76, 18.16, 11.44; exact mass: 444.3607 (444.3604 calcd for $C_{29}H_{48}O_3$).

7β-Acetoxycholest-5-en-3β-ol (88)[369, 399, 400]

The method for conversion of **87** to **89** was used. Thus, **86** (306 mg, 547 μmol) afforded 240 mg (99%) of **88** as a white solid: mp 89 - 90 °C, (lit.[400] mp 83 - 84 °C), (lit.[399] mp 131 - 133 °C); $[\alpha]_D^{24}$ +65.8° (*c* 1.15, $CHCl_3$), (lit.[399] $[\alpha]_D^{24}$ +76° (*c* 0.9, $CHCl_3$)), (lit.[400] $[\alpha]_D$ +72.5° ($CHCl_3$)); IR ($CHCl_3$ cast) 3390 (br, O-H), 2948, 2870 (C-H), 1734 (C=O), 1240 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.20 (dd, 1.6, 1.6 Hz, 1H, C<u>H</u>=C), 5.01 (ddd, 8.6, 2.0, 2.0 Hz, 1H, C<u>H</u>OAc), 3.54 (dddd, 10.8, 10.8, 4.4, 4.4 Hz, 1H, C<u>H</u>OH), 2.28 (m, 2H, 4-C<u>H</u>$_2$), 2.02 (s, 3H, C<u>H</u>$_3$CO$_2$), 1.9-2.1 (m, 2H, C<u>H</u>, C<u>H</u>$_2$), 0.9-1.9 (m, 23H, C<u>H</u>, C<u>H</u>$_2$), 1.071 (s, 3H, C<u>H</u>$_3$-19), 0.912 (d, 6.5 Hz, 3H, C<u>H</u>$_3$-21), 0.864 (d, 6.0 Hz, 3H, C<u>H</u>$_3$-26), 0.859 (d, 6.5 Hz, 3H, C<u>H</u>$_3$-27), 0.692 (s, 3H, C<u>H</u>$_3$-18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.09 (wk, <u>C</u>=O), 145.29 (wk, <u>C</u>=CH), 121.42 (<u>C</u>H=C), 75.80 (<u>C</u>HOAc), 71.16 (<u>C</u>HOH), 55.63, 55.56, 48.35, 42.90, 41.73, 39.50, 39.48, 36.92, 36.64, 36.48, 36.19, 35.64, 31.52, 28.32, 27.96, 25.16, 23.83, 22.72, 22.50, 21.55, 21.14, 19.02, 18.79, 11.78; exact mass: 444.3602 (444.3602 calcd for $C_{29}H_{48}O_3$). Anal. calcd for $C_{29}H_{48}O_3$: C, 78.33; H, 10.88. Found: C, 78.73; H, 10.69.

Isomerization of 84 or 85 with diethylaminosulfur trifluoride (DAST)

A modification of the methods of Markovskij[365] and of Middleton[366] was used. To a suspension of **84** (42.1 mg, 81.5 μmol) in hexane (1 mL) at -78 °C was added DAST (12.5 μL, 100 μmol). The mixture was allowed to warm to room temperature, and stirred for 30 min; when concentrated *in vacuo*, the residue had ^{19}F NMR (376 MHz, $CDCl_3$) δ -127.65 (dd, 43, 19 Hz, 20%), -168.20 (br d, 50 Hz, 13%), -176.40 (ddd, 50, 29, 10 Hz,

in vacuo. Column chromatography (EtOAc 4%/hexane 96%) gave 33.2 mg (79%) of a mixture of **84** and **85**, in a ratio of 20:80 by ^{1}H NMR. On a larger scale, pure **85** (23%) was collected, along with fractions containing a mixture of **84** and **85**.

In a similar manner **85** (10.0 mg, 19.3 μmol) afforded a mixture having ^{19}F NMR (376 MHz, $CDCl_3$) as above, with signals in a ratio of 42:17:41; work-up (as above) gave a mixture of **84** and **85** in a ratio of 17:83 by ^{1}H NMR. The reaction could also be used on a mixture of **84** and **85** directly after reduction of **83**.

7α-(2'-diazo-3', 3', 3'-trifluoropropionyloxy)-3β-(*t*-butyldimethylsilyl-oxy)-cholest-5-ene (90)

Acid chloride **75** (50 μL, 428 μmol) was added to a stirred solution of **85** (76.9 mg, 149 μmol) and dimethylaminopyridine (53 mg) in CH_2Cl_2 (10 mL). When the initial orange color faded to yellow (5 - 10 min), a further portion of **75** (30 μL) was added. This was repeated twice more until a persistent orange color resulted. The mixture was concentrated *in vacuo*. Flash chromatography (EtOAc 4%/hexane 96%) afforded 72.5 mg (75%) of **90** which was approximately 80% pure, as a gum: $[\alpha]_D^{24}$ -131° (*c* 0.83, $CHCl_3$); IR ($CHCl_3$ cast) 2953, 2937, 2867, 2857 (C-H), 2133 (C=N$^+$=N$^-$), 1725 (C=O ester), 1369, 1355, 1306, 1140, 1098, 837 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.58 (dd, 5.2, 1.0 Hz, 1H, C<u>H</u>=C), 5.50 (impurity), 5.1-5.35 (impurity), 5.00 (dd, 4.0, 4.0 Hz, 1H, C<u>H</u>OCO), 3.50 (dddd, 10.4, 10.4, 5.6, 5.6 Hz, 1H, C<u>H</u>OTBDMS), 2.1-2.4 (m, 2H, 4-C<u>H</u>$_2$), 1.0-2.1 (m, 24H, C<u>H</u>, C<u>H</u>$_2$), 0.975 (s, 3H, C<u>H</u>$_3$-19), 0.895 (d, 7.0 Hz, 3H, C<u>H</u>$_3$-21), 0.865 (s, 9H, (C<u>H</u>$_3$)$_3$CSi), 0.840 (2 X d, obscure, 6H, C<u>H</u>$_3$-26, C<u>H</u>$_3$-27), 0.643 (s, 3H, C<u>H</u>$_3$-18), 0.035 (s, 6H, (C<u>H</u>$_3$)$_2$Si); ^{19}F NMR (376 MHz, $CDCl_3$) δ -57.20 (br s, 81% of total ^{19}F signal); MS (NH_3-CI) m/e 624 (M$^+$ - N_2, 0.2%), 498 (M$^+$ - $CF_3CN_2CO_2H$, 0.6%), 367 (M$^+$ - $CF_3CN_2CO_2H$ - TBDMSOH, 40%).

Cholest-5-ene-3β,7α-diol (91)[368, 371]

The method[362] for conversion of **87** to **89** was used. Thus, **85** (55.0 mg, 106 μmol) afforded, after column chromatography (MeOH 2%/$CHCl_3$ 98%), 39.1 mg (91%) of **91** as a white solid: mp 154 - 155 °C, (lit.[371] mp 158 °C), (lit.[368] mp 182 - 184 °C); $[\alpha]_D^{24}$ -93.9° (*c* 1.95, $CHCl_3$), (lit.[371] $[\alpha]_D$ -13° ($CHCl_3$)), (lit.[368] $[\alpha]_{578}$ -75.8°); IR ($CHCl_3$ cast) 3270 (br, O-H), 2955, 2867 (C-H), 1465, 1060 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.60 (dd, 5.5, 1.3 Hz, 1H, C<u>H</u>=C), 3.85 (br m, 1H, 7-C<u>H</u>OH), 3.58 (m, 1H, 3-C<u>H</u>OH), 2.2-2.4 (m, 2H, 4-$C\underline{H}_2$), 0.9-2.1 (m, 26H, C<u>H</u>, $C\underline{H}_2$), 1.003 (s, 3H, $C\underline{H}_3$-19), 0.927 (d, 7 Hz, 3H, $C\underline{H}_3$-21), 0.870 (d, 7 Hz, 3H, $C\underline{H}_3$-26), 0.863 (d, 7 Hz, 3H, $C\underline{H}_3$-27), 0.673 (s, 3H, $C\underline{H}_3$-18); ^{13}C NMR (75 MHz, $CDCl_3$) δ 146.28 (wk, <u>C</u>=CH), 123.86 (<u>C</u>H=C), 71.26 (3-<u>C</u>HOH), 65.39 (7-<u>C</u>HOH), 55.91, 49.44, 42.29, 42.18, 42.07, 39.56, 39.22, 37.55, 37.43, 37.07, 36.21, 35.80, 31.37, 28.31, 28.04, 24.33, 23.76, 22.84, 22.60, 20.74, 18.78, 18.28, 11.67; exact mass: 402.3502 (402.3506 calcd for $C_{27}H_{46}O_2$).

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